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(54) Title: DNA DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT PHOSPHORYLATION SITES AND ANTIBODIES THERETO

(57) Abstract: The identification and use of two major DNA-PKcs autophosphorylation sites, Threonine (T) 2609 and Serine (S) 2056, including antibodies specific for phosphorylated T2609 and 52056. Peptides and polynucleotides encoding same, that feature these two sites of phosphorylation. The antibodies do not bind to the unphosphorylated DNA-PKcs protein or peptide, thus providing diagnostic tools to monitor the effectiveness of treatments which target the DNA repair pathway of cancer cells, and the ability to intervene or inhibit in phosphorylation of T2609 or 52056, either through application of a drug or an antibody, to increase the radiosensitivity of cancer cells.



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**DNA DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT
PHOSPHORYLATION SITES AND ANTIBODIES THERETO**

CROSS-REFERENCE TO RELATED APPLICATIONS

5 [001] This application claims priority to U.S. Provisional Patent Application No. 60/375,094, which was filed on April 22, 2002, which is incorporated by reference in its entirety.

STATEMENT REGARDING SEQUENCE LISTING

10 [002] Applicants assert that the attached paper copy of the Sequence Listing for the utility application, "DNA Dependent Protein Kinase Catalytic Subunit Phosphorylation Sites and Antibodies Thereto," claiming priority to U.S. Provisional Patent Application No. 60/375,094, filed on April 21, 2003, is identical to the Sequence Listing in computer readable form found on the accompanying computer disk, as required by 37 CFR 1.821(c) and is hereby incorporated by reference in its entirety.

15

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

20 [003] This invention generally relates to the field of cancer treatment, therapeutics and diagnostics. More specifically, the invention describes antibodies and a method useful for increasing the radiation sensitivity of cancer cells. The invention also provides methods of designing inhibitors of DNA-PKcs that are more specific and result in less harmful side effects.

DESCRIPTION OF THE RELATED ART

25 [004] In the clinical setting, the two most common treatments for cancer patients are a drug regimen or treatment with high doses of radiation, or a combination of both. Both approaches kill cancerous (and healthy) cells through a common mechanism of inducing DNA damage. DNA double-strand breaks (DSB) are the most common type of DNA damage resulting from either treatment. In human cells, DNA DSBs are repaired mainly by
30 the non-homologous end-joining pathway (NHEJ). The DNA-dependent protein kinase complex (DNA-PK) is a key player in the repair of DNA DSBs by this pathway. If DNA-PK is defective, cells are unable to repair DNA DSBs, and thus become highly sensitive to the

effects of ionizing radiation and of various cancer drugs. Since DNA-PK is a protein kinase, it is able to transfer phosphate groups to target proteins, and thereby regulate their function. DNA-PK is a protein complex consisting of its DNA-binding and regulatory subunit, which is the Ku protein, and the catalytic subunit, called DNA-PKcs. In the presence of DNA DSBs, Ku binds to the ends of the DNA and recruits DNA-PKcs to the site of the DSB. Once bound to Ku and DNA, DNA-PKcs becomes activated and is capable of phosphorylating target proteins.

[005] Although the biochemical properties of DNA-PK have been extensively studies *in vitro*, very little is known about how DNA-PK functions *in vivo* in relation to the repair of DNA DSBs. This lack of progress in studying the physiological functions of DNA-PK is in part due to the unavailability of the right tools or assays to evaluate DNA-PK *in vivo* activity.

[006] Currently, one of the most commonly used methods to study DNA repair proteins is by immunofluorescence with an antibody to the protein of interest. In response to DNA damage, many of the DNA repair proteins form "foci" that can be visualized with antibodies. It is generally believed that these DNA damage-induced foci correspond to sites where the damages DNA is actively being repaired.

[007] It is currently not possible to detect DNA-PK foci with the antibodies available because DNA-PK is quite abundant in the nucleus, thus when one performs immunofluorescence with any of the available antibodies, the entire nucleus will produce a signal, making it impossible to see any discernable foci. Therefore, it is of interest to develop an antibody that can overcome the problem associated with a very high background signal and can recognize the phosphorylated form of DNA-PKcs when bound to site of DNA DSBs.

[008] DNA-PK is a serine/threonine protein kinase that *in vitro* is activated by DNA ends and has long been established to play an important role in the repair of DNA double-strand breaks (DSB) by the NHEJ pathway (Smith and Jackson, *Genes Dev.* 1999 Apr 15;13(8):916-34). DNA-PK is capable of autophosphorylating the two Ku subunits, Ku70 and Ku80 according to Chan et al., *Biochemistry* 1999 Feb 9;38(6):1819-28. Autophosphorylation of DNA-PKcs causes it to dissociate from Ku, resulting in the loss of kinase activity (Chan and Lees-Miller, *J Biol Chem.* 1996 Apr 12;271(15):8936-41). In addition, the inventors have shown that the kinase activity of DNA-PKcs is absolutely required for its function in the NHEJ pathway since a DNA-PKcs-deficient CHO cell line expressing a kinase dead form of DNA-PKcs was incapable of repair (Kurimasa et al., *The Journal of Immunology*, 2000, 165: 3883-3889). Therefore, the kinase activity of DNA-PK is

absolutely required for the repair of DNA DSBs; however, the molecular mechanism of this requirement for kinase activity remains to be elucidated. DNA-PK is also capable of autophosphorylation, that is, it transfers phosphate groups onto itself, and that autophosphorylation may be an important mechanism for regulating its kinase activity (Kurimasa et al., *Molecular and Cellular Biology*, May 1999, p. 3877-3884, Vol. 19, No. 5).

[009] DNA-PKcs is an extremely large protein consisting of 4129 amino acids, and therefore identifying the site of autophosphorylation is comparable to finding a very small needle in a large haystack. Cloning of the DNA-PKcs cDNA is difficult, since the cDNA exceeds 13 kb. In the past, using classical biochemical techniques, several labs have attempted but failed to identify the autophosphorylation sites. For example, *in vivo* radiolabelling with ^{32}P and 2-dimensional phosphopeptide mapping failed to identify any autophosphorylation sites.

[010] One goal of radiation biology is to find ways to increase the radiation sensitivity of cancer cells. If this could be achieved, it would then be possible to treat cancer patients with lower doses of radiation and thereby dramatically decrease the side effects and complications associated with radiation treatment.

[011] If the site of phosphorylation in DNA-PK could be specifically blocked in cancer cells, for example with a DNA-PKcs inhibitor, then this should inhibit DNA-PKcs-mediated repair of DNA DSBs and thereby increase the radiation sensitivity of the treated cancer cells. Another possible means of increasing radiation sensitivity is the development of therapeutic antibodies that can specifically recognize and bind to the phosphorylated protein.

BRIEF SUMMARY OF THE INVENTION

[012] The present invention is directed to the identification and use of two major DNA-PKcs autophosphorylation sites, Threonine (T) 2609 and Serine (S) 2056, including antibodies specific for phosphorylated T2609 and S2056. It is demonstrated that phosphorylation of these sites, carried out *in vivo* by the DNA-PKcs itself (i.e. autophosphorylation), is required for DNA-PK activity and, furthermore, that such activity repairs double strand DNA breaks (DSBs) and improves cell survival to ionizing radiation (IR). For example, it is demonstrated a point mutation at position 2056 from serine to alanine and position 2609 from threonine to alanine results in cells that are radiosensitive.

[013] The present invention further provides phosphospecific antibodies that recognize these specific sites of phosphorylation in DNA-PKcs. The antibodies do not bind to the unphosphorylated DNA-PKcs protein or peptide. This provides diagnostic tools based

on the ability to identify the phosphorylation status of the DNA-PKcs autophosphorylation sites. One can monitor the effectiveness of treatments which target the DNA repair pathway of cancer cells, such as radiation treatment and inhibitor drugs. Also, the ability to intervene in autophosphorylation of T2609 or S2056, either through application of a drug or an antibody, would increase the radiation-induced killing of cancer cells.

[014] In one embodiment there is provided an antibody which specifically binds to an epitope defined by at least a ten amino acid sequence from DNA-PKcs and comprising a phosphorylated threonine at position T2609 in human DNA-PKcs, which antibody does not bind when T2609 is not phosphorylated. The antibody may be an affinity purified polyclonal antibody or a monoclonal antibody. The monoclonal may be a conventional hybridoma produced mouse monoclonal, or may be a human monoclonal produced by known techniques. In one embodiment, the human monoclonal is produced using a mouse with a human immune system as an immune cell donor in a hybridoma process. One specific embodiment is the pT2609 monoclonal antibody, pT2609mAb.

[015] The invention further comprises an antibody which specifically binds to an epitope defined by at least a ten amino acid sequence from DNA-PKcs and comprising a phosphorylated serine at position S2056 in human DNA-PKcs, which antibody does not bind when S2056 is not phosphorylated. Again, the antibody may be an affinity purified polyclonal antibody or a monoclonal antibody. The monoclonal may be a conventional hybridoma produced mouse monoclonal, or may be a human monoclonal produced by known techniques. In one embodiment, the human monoclonal is produced by using a mouse with a human immune system as an immune cell donor in a hybridoma process. One specific embodiment is the pS2056 monoclonal antibody, pS2056mAb.

[016] In any case, the binding epitope is contained both on full length DNA-PKcs and subsequences thereof, said subsequences having at least about 10 amino acids.

[017] The present invention further comprises methods for determining the ability of a test compound to block phosphorylation of human DNA-PKcs. One method comprising the following steps: (a) providing a sample containing a DNA-PKcs peptide fragment capable of being phosphorylated; (b) adding the test compound to the sample; (c) inducing phosphorylation of the DNA-PKcs protein in the sample; and (d) measuring the resulting phosphorylation of DNA-PKcs at T2609 or S2056 in the presence of the test compound. This is preferably done in comparison to a sample containing a DNA-PKcs peptide fragment which is phosphorylated in the absence of the test compound.

[018] The method may also involve providing a sample containing an artificial peptide containing the T2609 and/or S2056 site. Recombinant DNA-PK or DNA-PKcs is added to the mixture and will phosphorylate the artificial peptide. The artificial peptide may be on the order of about 1000 amino acids long or as short as 20 amino acids long.

5 [019] The measuring step may be carried out by measuring the binding of an antibody which specifically binds to an epitope comprising either or both of (a) a phosphorylated serine at position S2056 in human DNA-PKcs or (b) a phosphorylated threonine at position T2609 in human DNA-PKcs

[020] Known kinase inhibitors provide suitable starting points for assaying test
0 compounds that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. In this assay, test compounds are any organic molecules that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. Non-limiting examples include wortmannin, substituted or unsubstituted imidazoles, pyrazoles, benzofluoranthenes, thiazoles, isoquinolinones, dihydrolisoquinolinones, phthalazinones and related compounds and derivatives thereof. For
5 example, since wortmannin has been shown herein to inhibit the phosphorylation of T2609 and S2056, derivatives and analogs of wortmannin provide sources of test compounds to be tested in the present assay. Functional groups could be introduced into the wortmannin structure adjacent to the heterocyclic oxygen adjacent to C21 or the double bond between C4 and C21.

20 [021] To identify drug inhibitors of DNA-PK, one first initially screens available chemical libraries for test compounds that could inhibit DNA-PK kinase activity *in vitro* or organic molecules that are capable of blocking or inhibiting phosphorylation of DNA-PKcs. The compounds in these chemical libraries can be added to *in vitro* DNA-PK kinase assays to identify the ones that could inhibit DNA-PK activity. Because phosphorylation of T2609 and
25 S2056 is via an autophosphorylation mechanism, it can be speculated that any drug compounds that inhibit the kinase activity will inhibit the autophosphorylation of these two sites. Once these compounds have been identified, cellular studies can then be carried out to evaluate their efficacy.

[022] Further aspects of the present invention involve phosphopeptides that have
30 been prepared for use in injection into animals in the course of antibody preparation (haptens) or for use as artificial phosphorylation substrates. These peptides will have less than 30 amino acids and comprise SEQ ID NO: 1 or SEQ ID NO: 2, or sequences having at least 90% homology thereto having the requisite serine or threonine residues, preferably in an SQ or TQ

sequence. These phosphopeptides may also have an amino acid other than the wild type T2609 or S2056, to serve as negative controls.

[023] The above described isolated peptides have further utility when T2609 and/or S2056 is replaced by an amino acid which is not phosphorylated, such as: Valine, Alanine, Glycine, or Leucine. These embodiments serve as negative controls and will inhibit phosphorylation.

[024] The above described peptides may be encoded by an isolated polynucleotide cloned and inserted into a suitable host vector.

[025] The present invention further comprises a method of measuring radiosensitivity of cells in a subject undergoing radiation treatment. This method comprises the steps of (a) providing a cell sample from said subject and containing DNA-PKcs, for example a blood sample or a tissue sample from the irradiated area (e.g. a nuclear extract may be prepared from this sample); (b) adding to said sample a labeled antibody which binds to phosphorylated residue T2609 or phosphorylated residue S2056 but not the unphosphorylated residues; (c) removing unbound antibody from the sample, such as by washing, as is known in the art; and (d) measuring the degree of phosphorylation of the DNA-PKcs by determining the amount of antibody bound to the DNA-PK in the sample. The degree of antibody binding to DNA-PKcs in the cell sample correlates to the degree of phosphorylation, a higher degree of phosphorylation indicating less radiation sensitivity

[026] Since most treatment for cancer entails inducing DNA damage, a pT2609 or pS2056 antibody can be a very useful diagnostic tool for determining the efficacy of the treatment. For example, the antibody can be used to confirm that the cancer treatment is indeed causing DNA damage in the cancer cells; conversely, the antibody can be used to determine the effects of the treatment on healthy cells.

BRIEF DESCRIPTION OF THE DRAWINGS

[027] Fig. 1 is a mass spectra of sequencing of the *in vitro* phosphorylated DNA-PKcs peptide that was first identified by MALDI-TOF mass spectrometry. The plot shows a plot of relative intensity vs the mass-to-charge ratio (m/z) of the phosphorylated peptide having the shown sequence from 2599 to 2619. Assignment of all the mass spectra peaks unequivocally identify T2609 as the site of phosphorylation.

[028] Fig. 2 is the mass spectra of phosphatase-treated (bottom trace) and the mock treated (top trace) DNA-PKcs peptides from irradiated HeLa cells which were purified by immunoprecipitation and digested with Asp-N protease, then analyzed by MALDI-TOF mass

spectrometry. The loss of the peak with the m/z of 3511 with phosphatase treatment, and the presence of the peak corresponding to the unphosphorylated peptide (m/z of 3433) allowed the positive assignment of a phosphorylation site to the sequence of DNA-PKcs between amino acids 2044-2072.

5 [029] Fig. 3A is a Western blot showing DNA-PKcs protein expression levels of vector along (V3-JM), full-length wild-type DNA-PKcs (V3-F18) and T2609A mutant of DNA-PKcs in V3 cell line (top). Hamster Werner proteins were analyzed to show equal sample loading (bottom). Fig. 3B is a graph showing that the T2609A, S2056A and the T2609A/S2056A double mutation increase cellular radiation sensitivity. Fig. 3C is a graph
10 showing that the T2609A and S2056A mutations compromise DSB repair in cells as determined by the fraction of activity released (FAR) assay.

[030] Fig. 4A is two Western blots showing that the pT2609pAb is specific for the phosphorylated T2609 site in wild type DNA-PKcs. Fig. 4B is two Western blots showing that the pT2609 polyclonal antibody (pT2609pAb) does not recognize unphosphorylated
15 DNA-PKcs at the molar ratios given as compared to 25-4 DNA-PKcs monoclonal antibody (25-4 mAb). Fig. 4C is two Western blots showing that the pS2056 polyclonal antibody (pS2056pAb) does not recognize unphosphorylated S2056 in DNA-PKcs as compared to 25-4 DNA-PKcs monoclonal antibody (25-4 mAb).

[031] Fig. 5A is a Western blot of HeLa cell nuclear extracts, probed with pT2609 antibody (upper panel) and 25-4 monoclonal antibody to DNA-PKcs (bottom panel), after
20 either mock-treatment or irradiation with 10 Gy and recovery for various times. Fig. 5B is a Western blot of HeLa cell nuclear extracts, probed with pT2609pAb (upper panel) and 25-4 monoclonal antibody to DNA-PKcs (bottom panel), after irradiation at the indicated dose and recovery for 30 minutes. Fig. 5C is a Western blot showing that pT2609 polyclonal antibody
25 can be used to immunoprecipitate phosphorylated DNA-PKcs from unirradiated HeLa nuclear extract (lane 1) but not unphosphorylated DNA-PKcs from extracts made from HeLa cells irradiated with 25 Gy and harvested after 30 min recovery period. The 25-4 monoclonal antibody does not discriminate between phosphorylated or unphosphorylated DNA-PKcs. Fig. 5D shows by Western blot that phosphorylation of T2609 in response to DNA damage
30 can be inhibited with wortmannin treatment (left panel) and is inducible in A-T cells (right panel).

[032] Fig 6A is a Western blot showing that phosphorylation of S2056 is IR-inducible. Fig. 6B is a time course Western blot of IR-inducible phosphorylation of Ser 2056.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

A. Definitions

- [033] "Radiosensitization" herein refers to a means of increasing the sensitivity of human cells to the effects of ionizing radiation and cancer drugs that induce DNA double-strand breaks (DSBs). By increasing the radiosensitivity of cancer cells, patients can be treated with lower doses of radiation or chemotherapeutic regimen and thereby decrease the harmful side effects of the treatment.
- [034] "Gy" herein refers to describe the unit (SI unit) of absorbed dose of radiation (Gy), wherein $1 \text{ Gy} = 1 \text{ J kg}^{-1} = 100 \text{ rad}$.
- [035] "Epitope" has its conventional meaning, i.e. a single antigenic determinant. Functionally it is the portion of an antigen (e.g. a PKcs peptide) which combines with the antibody paratope. Structurally, it is the specific amino acid residues or portions thereof to which an anti-peptide antibody binds.
- [036] "DNA-PKcs" herein refers to DNA-dependent protein kinase catalytic subunit (EC 2.7.1.37), preferably human DNA-PKcs. As used herein, the numbering is based on GenBank Accession Number P78527, as set out in SEQ ID NO: 3. Specifically, T2609 is threonine 2609 in Genbank Accession Number P78527. S2056 is serine 2056 in GenBank Accession Number P78527. DNA-PK refers to the entire enzyme. DNA-PKcs is encoded by the nucleotide sequence as set out in SEQ ID NO: 15, having GenBank Accession Number U47077.
- [037] "Monoclonal antibody" has its conventional meaning, and is explained more fully in U.S. patent 4,619,895, hereby incorporated by reference for purposes of describing preparation and characterization of mouse monoclonal antibodies, U.S. patent 4,744,982 hereby incorporated by reference for purposes of describing human/human monoclonal antibody preparation and characterization, U.S. patent 5,874,540, hereby incorporated by reference for purposes of describing the preparation and characterization of CDR-grafted humanized antibodies, and U.S. patent 6,075,181, hereby incorporated by reference for purposes of describing the preparation and characterization of human antibodies derived from immunized xenomice.
- [038] The abbreviation "mAb" herein refers to monoclonal antibodies and the abbreviation "pAb" herein refers to polyclonal antibodies.

[039] "Humanize," when applied to antibodies, herein refers to methods of generating human monoclonal antibodies, as exemplified by van de Winkel, in U.S. Pat. No. 6,111,166, hereby incorporated by reference for purposes of describing such methods.

5 [040] "Isolated," when applied to a polynucleotide, herein refers to that the polynucleotide has been removed from its natural genetic milieu and is thus free of the extraneous or unwanted coding sequences, and is in a form suitable for use within genetically engineered protein production systems. Such isolated molecules are those that are separated from their natural environment and include cDNA, synthetic DNA and genomic clones. Isolated DNA molecules of the present invention are free of other genes with which they are
10 ordinarily associated, but may include naturally occurring 5' and 3' untranslated regions such as promoters and terminators. The identification of associated regions will be evident to one of ordinary skill in the art (see for example, Dynan and Tijan, *Nature* 316:774-78, 1985).

[041] "Isolated," when applied to a polypeptide or protein, herein refers to a polypeptide or protein that is found in a condition other than its native environment, such as
15 apart from blood and animal tissue. In a preferred form, the isolated polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin. It is preferred to provide the polypeptides in a highly purified form, i.e. greater than 95% pure, more preferably greater than 99% pure. When used in this context, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as
20 dimers or alternatively glycosylated or derivatized forms.

[042] "Polynucleotide" herein refers to a single- or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end. Polynucleotides include RNA and DNA, and may be isolated from natural sources, synthesized in vitro, or prepared from a combination of natural and synthetic molecules. Sizes of polynucleotides are
25 expressed as base pairs (abbreviated "bp"), nucleotides ("nt"), or kilobases ("kb"). Where the context allows, the latter two terms may describe polynucleotides that are single-stranded or double-stranded. When the term is applied to double-stranded molecules it is used to denote overall length and will be understood to be equivalent to the term "base pairs". It will be recognized by those skilled in the art that the two strands of a double-stranded polynucleotide
30 may differ slightly in length and that the ends thereof may be staggered as a result of enzymatic cleavage; thus all nucleotides within a double-stranded polynucleotide molecule may not be paired. Such unpaired ends will in general not exceed 20 nt in length.

[043] "Polypeptide" herein refers to a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides".

[044] "Homologous" herein refers to the sequence similarity between two polymeric molecules, e.g., between two nucleic acid molecules, e.g., two DNA molecules or two RNA molecules, or between two peptide molecules. When a subunit position in both of the two molecules is occupied by the same monomeric subunit, e.g., if a position in each of two DNA molecules is occupied by adenine, then they are homologous at that position. The homology between two sequences is a direct function of the number of matching or homologous positions, e.g., if half (e.g., five positions in a polymer ten subunits in length) of the positions in two compound sequences are homologous then the two sequences are 50% homologous, if 90% of the positions, e.g., 9 of 10, are matched or homologous, the two sequences share 90% homology. By way of example, the DNA sequences 3' ATTGCC 5' and 3' TATGCG 5' share 50% homology. Any of a variety of known algorithms may be used to calculate the percent homology between two nucleic acids or two proteins of interest and these are well-known in the art.

[045] "Substantial homology" or "substantial identity", when referring to polypeptides, herein refers to that the polypeptide or protein in question exhibits at least about 30% identity using BLASTP (Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410) with an entire naturally-occurring protein or a portion thereof, usually at least about 70% identity over the common lengths, more usually at least about 80% identity, preferably at least about 90% identity, and more preferably at least about 90% identity or 90% positive, whichever is less. For purposes of calculating homology between two polypeptides, the standard BLASTP 2.2.5 defaults are used, namely "Expect 10," "Word size 3," "BLOSUM62 Matrix" and "Gap Costs Existence10, Extension 1."

[046] In this specification, the singular forms "a," "an," and "the" include plural reference unless the context clearly dictates otherwise. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which this invention belongs.

B. Role of DNA PK-cs in Double Strand Break Repair

[047] Repair of DNA double strand breaks (DSBs) in mammalian cells is mainly mediated by the non-homologous end-joining pathway (NHEJ). The DNA-dependent protein kinase (DNA-PK) complex play critical roles in the NHEJ pathway since mammalian

cell lines that lack components of the complex show severe radiation sensitivity and DNA repair defects. DNA-PK is a serine/threonine protein kinase that is activated by the DNA ends *in vitro* and is composed of the DNA-binding and regulatory subunit, Ku, and the catalytic subunit, DNA-PKcs. DNA-PK kinase activity is required for the repair of DNA DSBs *in vivo* (Kurimasa et al., *Mol Cell Biol.* 1999 May; 19(5):3877-84). Previously, it was demonstrated that DNA-PK activity is negatively regulated by an autophosphorylation mechanism *in vitro*. (Chan et al., *Biochem Cell Biol* 74: 67-73, 1996).

[048] In the preferred embodiment, the DNA-PKcs being investigated has an amino acid sequence comprising SEQ ID NO: 3 and wild-type cDNA sequence comprising SEQ ID NO: 15.

[049] Herein is reported the method and identification of residues of DNA-PKcs which can act as major *in vitro* and *in vivo* autophosphorylation sites.

C. Autophosphorylation Sites in DNA-PKcs

[050] Determination of autophosphorylation sites in DNA-PKcs is preferably done by mass spectrometry. For example, purified DNA-PKcs and Ku can be autophosphorylated by adding a low concentration of ATP to allow phosphorylation of the most preferential site and then the autophosphorylated DNA-PKcs was analyzed by mass spectrometry. Alternatively, DNA-PKcs can be immunoprecipitated from nuclear extracts prepared from irradiated HeLa cells and then analyzed by mass spectrometry.

[051] As described below, two major *in vitro* and *in vivo* autophosphorylation sites of the residues of T2609 and S2056 were identified by mass spectrometry. Purified DNA-PKcs and Ku were autophosphorylated with low concentration of ATP (50uM) to allow phosphorylation of the most preferential site. Referring now to Fig. 1, the *in vitro* autophosphorylated DNA-PKcs was analyzed by mass spectrometry and T2609 was unambiguously identified as a major site of autophosphorylation. T2609 lies in a region of DNA-PKcs that is not conserved between the various members of the phosphatidylinositol 3-kinase (PI-3) family members, to which DNA-PKcs is a member of. However, T2609 was absolutely conserved in all known DNA-PKcs homologues found in GenBank (i.e. mouse, dog, horse, chicken and xenopus) when the sequences are compared. This suggests the significance of the phosphorylation of this residue and that phosphorylation of DNA-PKcs at this residue may be conserved throughout evolution.

[052] Referring now to Fig. 2, a second major *in vitro* and *in vivo* autophosphorylation site of DNA-PKcs, S2056, was identified by immunoprecipitating DNA-PKcs from nuclear extracts prepared from irradiated HeLa cells and analyzed by mass

spectrometry. As described in detail below, mass spectrometry identified the following phosphopeptide sequence, DFSTGVQSYSSYSSQDPRPATGRFRRREQR (SEQ ID NO: 5), which corresponds to amino acids 2044 to 2072 of DNA-PKcs (S2056 is underlined). Upon careful analysis of the sequence, S2056 proved to be the site of phosphorylation. This is consistent with prior suggestions that DNA-PK preferentially phosphorylates "SQ" and "TQ" sequences and S2056 followed this "SQ" consensus sequence. Similar to T2609, the sequence alignment with other vertebrate DNA-PKcs homologues in GenBank shows that amino acids 2044 to 2056 in DNA-PKcs are highly conserved throughout evolution in vertebrates.

D. Role of DNA-PKcs Autophosphorylation Sites in Radiation Sensitivity

[053] To investigate the biological significance of the autophosphorylation of these DNA-PKcs sites in relation to DNA repair, wild-type or mutant DNA-PKcs having the site of autophosphorylation mutated were transfected into the DNA-PKcs-defective V3 cell line (Kurimasa et al., *J Immunol.* 2000 Oct 1;165(7):3883-9). The resulting V3 cell lines were isolated and evaluated for DNA-PKcs protein expression levels, radiation sensitivity, and DNA repair defects. Cells expressing the mutant DNA-PKcs protein exhibit a more severe radiation sensitivity phenotype as compared with wild-type DNA-PKcs protein yet not as severe as the V3 cell line that totally lacks DNA-PKcs. The V3-mutant DNA-PKcs cells exhibits a radiation sensitivity phenotype of about a 10 fold increase in cell death at 5 Gy when compared with V3-wild type cells, demonstrating a dramatic difference in radiation sensitivity in mammalian cells.

E. Generation of anti-pT2609 and anti-pS2056 phosphospecific antibodies

[054] To study the *in vivo* phosphorylation status of DNA-PKcs at the autophosphorylation sites, a phosphospecific antibody that recognizes the phosphorylated residue of DNA-PKcs is generated, then affinity purified to insure specificity.

[055] DNA-PKcs phosphospecific antibodies can be made by a number of methods known in the art. These phosphospecific antibodies include antibodies which recognize phosphorylated T2609, herein referred to as pT2609 antibodies and phosphorylated S2056, herein referred to as pS2056 antibodies. A preferred method is by generating phosphopeptides. These phosphopeptides can be synthesized or produced by first amplifying and cloning cDNA fragments of SEQ ID NO: 15, the cDNA sequence of human DNA-PKcs (GenBank Accession No. U47077), and then expressing peptide fragments of DNA-PKcs from the cloned cDNAs. These phosphopeptide fragments include the site of

autophosphorylation and the adjacent DNA-PKcs amino acid sequence on either side of the position being autophosphorylated. It is preferred that at least 6, preferably no more than 10 amino acids of the wild-type DNA-PKcs protein sequence are used on either side of the phosphorylation site to generate very specific antibodies. Two such preferred phosphopeptides are SEQ ID NO: 1 and 2 and shown below.

SEQ ID NO: 1 N'--TPMFVET*QASQGTC--C' (* indicating phospho group at T2609)

SEQ ID NO: 2 N'--QSYSYSS*QDPRPAC--C' (* indicating phospho group at S2056)

[056] Since synthesized phosphopeptides are not always immunogenic on their own, the peptides were conjugated to a carrier protein before use. Appropriate carrier proteins include, but are not limited to, Keyhole limpet hemacyanin (KLH), bovine serum albumin (BSA) and ovalbumin (OVA). The conjugated phosphopeptides should then be mixed with adjuvant and injected into a mammal, preferably a rabbit through intradermal injection, to elicit an immunogenic response. Samples of serum can be collected and tested by ELISA assay to determine the titer of the antibodies and then harvested.

[057] Polyclonal pT2609 and pS2056 antibodies can be purified by passing the harvested antibodies through an affinity column. However, monoclonal antibodies are preferred over polyclonal antibodies and can be generated according to standard methods known in the art of creating an immortal cell line which expresses the antibody.

[058] Nonhuman antibodies are highly immunogenic in human thus limiting their therapeutic potential. In order to reduce their immunogenicity, nonhuman antibodies need to be humanized for therapeutic application. Through the years, many researchers have developed different strategies to humanize the nonhuman antibodies. One such example is using "HuMAb-Mouse" technology available from MEDAREX, Inc. (Princeton, NJ). "HuMAb-Mouse" is a strain of transgenic mice that harbors the entire human immunoglobulin (Ig) loci and thus can be used to produce fully human monoclonal pT2609 and pS2056 antibodies.

[059] Immunoblotting using the phosphospecific antibodies of the invention with unphosphorylated DNA-PKcs should not produce a detectable signal at preferably 0.5-10 fold molar excess (relative to the phosphorylated DNA-PKcs), more preferably at 50 fold molar excess and most preferably no signal is detected at even 100 fold molar excess.

F. Designing and Making DNA-PKcs Inhibitor Drugs

[060] The phosphorylation of DNA-PKcs at Threonine 2609 and Serine 2056 is required for the repair of DNA double strand breaks. By inhibiting the phosphorylation of

these two sites with small molecules, it may be possible to increase the radiation-induced killing of cancer cells.

[061] To identify drug inhibitors of DNA-PK, one first initially screens available chemical libraries for test compounds that could inhibit DNA-PK kinase activity *in vitro* or organic molecules that are capable of blocking or inhibiting phosphorylation of DNA-PKs. Analysis of known kinase inhibitors provides suitable starting points and non-limiting examples include wortmannin, substituted or unsubstituted imidazoles, pyrazoles, benzofluoranthenes, thiazoles, isoquinolinones, dihydrolisoquinolinones, phthalazinones and related compounds and derivatives thereof. The compounds in these chemical libraries can be added to *in vitro* DNA-PK kinase assays to identify the ones that could inhibit DNA-PK activity. Because phosphorylation of T2609 and S2056 is via an autophosphorylation mechanism, it can be speculated that any drug compounds that inhibit the kinase activity will inhibit the autophosphorylation of these two sites. Once these compounds have been identified, cellular studies can then be carried out to evaluate their efficacy.

[062] Amino acid peptide fragments of DNA-PKs around the T2609 and S2056 sites were expressed in *E. coli* despite the difficulty encountered in cloning the corresponding cDNA sequence. In a preferred embodiment, the DNA-PKs cDNA clones containing SEQ ID NO: 18 and SEQ ID NO: 22, which express peptide fragments corresponding to the amino acid sequence of DNA-PKs from residues 1879-2182 and from 2500-2700 respectively, are made. In another preferred embodiment, an 822 amino acid fragment (residues 1879-2700) can be expressed from a cDNA clone containing SEQ ID NO: 20 because this fragment once expressed encompasses both phosphorylation sites.

[063] These fragments which encompass one or both phosphorylation sites can be made by amplifying the appropriate cDNA sequence from a full-length DNA-PKs cDNA (SEQ ID NO: 15, GenBank Accession No. U47077) by PCR, then cloning and expressing the cDNA sequence to generate the peptide fragment. Primers can be designed and made from SEQ ID NO: 15. It is preferred that the peptide fragment containing the phosphorylation site, be of a length of at least 10, preferably 100, and more preferably about 1000 amino acids of the DNA-PKs protein sequence.

[064] In one embodiment, these fragments can be used to test how effectively potential drugs inhibit the phosphorylation and activation of DNA-PKs. Recombinant fragments containing these two phosphorylation sites can be used as molecular targets for small molecular screening. Specifically, small molecules which can bind to these fragments with high affinity will be identified. The inhibition capability of these small molecules can

be verified by their ability to block T2609 and S2056 phosphorylation. The radiation sensitization ability of these small molecules can then be verified in human cells upon radiation damage.

[065] In addition, fragments that may also be useful can be expressed from the following clones. These clones were made to express the following residues of DNA-PKcs from the indicated corresponding cloned cDNA sequence: 1879-2182 cDNA (SEQ ID NO: 18), 1879-2267 cDNA (SEQ ID NO: 19), 2261-2700 cDNA (SEQ ID NO: 21), 2275-2702 cDNA (SEQ ID NO: 23), 2429-2702 cDNA (SEQ ID NO: 24), 2561-2700 cDNA (SEQ ID NO: 25), and 2600-2702 cDNA (SEQ ID NO: 26).

G. Peptide Inhibitor Drugs

[066] One embodiment is to use the antibodies of the invention for use as an inhibitor of the phosphorylation and thereby block DNA repair which results in radiosensitization of cancer cells. Because of the specificity of the pT2609 and pS2056 antibodies of the invention, only the sites of phosphorylation are inhibited. Furthermore, phosphorylation of T2609 and S2056 occurs only in cells that have been irradiated and suffer DNA damage. Therefore, the use of the antibodies, or peptide fragments thereof, as DNA repair inhibitors will not affect other proteins or even other parts and functions of the DNA-PKcs protein. This specificity will result in not only radiosensitizing cancer cells, but also this antibody will reduce the other harmful side effects of inhibiting all DNA-PKcs function.

[067] If phosphorylation of T2609 is required for the recruitment of other proteins needed at the site of DNA DSBs, then another means of disrupting this step is to overexpress a small polypeptide spanning the region that surrounds T2609 or S2056 with an Aspartic acid mutation to simulate the phosphorylated state and create a "dominant negative" effect. Therefore, in another embodiment, a polypeptide, such as the peptides generated in Example 2, made with an Aspartic acid or other similarly negatively charged amino acid substituted at residue 2609 or 2056 to mimic the phosphorylated state of T2609, can be overexpressed or administered to compete with endogenous phosphorylated DNA-PKcs. This would "squench" DNA-PKcs function and therefore lead to an increase in radiation sensitivity.

H. Diagnostic Tools for Detecting Efficacy of Therapeutic Treatments

[068] Companies are developing specific inhibitors for DNA-PKcs or Ku for the purpose of sensitizing cancer cells for radiation therapy. Currently, there is no efficient way to estimate the amount of the inhibitors to be used for sensitization. Antibodies against T2609 or S2506 can be used as a diagnostic tool to effectively monitor blood samples in the test tube to estimate the dose to be used to effectively block the autophosphorylation of

DNA-PKcs. The present pT2609 or pS2056 antibodies can provide a diagnostic tool for determining the efficacy of treatment using DNA-PKcs or Ku inhibitors. The antibodies of the invention can be used to confirm whether the cancer treatment the patient is undergoing is indeed causing DNA damage in the cancer cells; conversely, the antibody can be used to determine the effects of the treatment on healthy cells. Furthermore, diagnostic tests to test the efficacy of inhibitors during drug development can be made based on observations of phosphorylation of T2609, such as in Example 6 or 8.

[069] In one embodiment, the antibodies of the invention can be used to determine the correct radiation dosage for each patient. The normal patient dosage is 2Gy/day up to 50 Gy/day. Since every cancer patient responds to radiation therapy differently, the pT2609 and pS2056 antibodies can be a very useful tool to monitor the effectiveness of the cancer treatment. In a specific embodiment, for example, a small blood sample is drawn from a cancer patient and a quick radiation pulse is applied to the sample to induce DNA damage, then contacting a small volume of the radiated blood with the antibodies of the invention. To increase the signal, the antibodies can be conjugated to another antibody or other means of detection used. Unbound antibodies are washed from the sample and antibodies bound to the patient's DNA-PKcs are measured. A large signal as compared to a control will indicate to an oncologist that any inhibitors that target DNA-PKcs are not working and not inhibiting DNA repair. That is, there is a high degree of DNA-PK autophosphorylation. A low or no signal would indicate that the inhibitors are working which has resulted in the radiosensitization of cancer cells. Alternatively, the blood sample is not irradiated but taken from a patient following radiation treatment to monitor the radiation therapy. In addition, the cells may be taken from a biopsy of the patient's tumor or cancer cells.

[070] In another embodiment, the peptide fragments of SEQ ID NOS: 4-14, would permit the screening of small molecular inhibitors to block phosphorylation at these sites. Small molecular inhibitors which would block the phosphorylation at T2609 or S2506 would be more effective radiosensitizers and have less side effects for radiotherapy. The reason is that the phosphorylation of these two sites only occurs after radiation or DNA damage in response to DSBs and only activated in DNA double-strand break repair. Therefore, inhibitors which are specific for these two sites should not have any effect in cells which have not been irradiated.

EXAMPLE 1

Determining T2609 and S2056 Sites of Autophosphorylation in DNA-PKcs by Mass Spectrometry

[071] First, purified human DNA-PKcs and Ku were autophosphorylated as previously described (Chan and Lees-Miller, *J Biol Chem* 271: 8936-8941, 1996), and hereby incorporated by reference, with the following change: 50 μ M ATP was used instead of 250 μ M to allow phosphorylation of the most preferential site. Purified DNA-PKcs and Ku proteins were preincubated at 30 °C. Reactions contained 25 mM Hepes, pH 7.5, 75 mM KCl, 10 mM MgCl, 1 mM dithiothreitol, 0.2 mM EGTA, 0.1 mM EDTA plus 10 μ g/ml sonicated calf thymus DNA, and 0.25 mM ATP containing stabilized [-P]ATP (Sigma Chemicals, St. Louis, MO) (specific activity, 500-1000 dpm/pmol) and were started by the addition of purified DNA-PK proteins (usually 0.05-0.1 μ g as indicated). Reactions were at 30 °C for 5-10 min and DNA-PK activity was calculated as nmol of phosphate incorporated into the peptide substrate per minute per milligram of protein. Unlabeled ATP or the nonhydrolyzable ATP analogue AMP-PNP (Sigma Chemicals, St. Louis, MO) were present where indicated at 50 μ M. After 0-10 min, aliquots were removed and analyzed by SDS-PAGE. The band corresponding to phosphorylated DNA-PKcs was excised and digested with trypsin.

[072] The tryptic DNA-PKcs fragments were analyzed by mass spectrometry as previously described by Zhang et al., *Anal Chem* 70: 2050-2059, 1998. This procedure facilitates the identification of precise phosphorylation sites in proteins separated by polyacrylamide gel electrophoresis by a combination of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF) and on-line capillary liquid chromatography electrospray tandem ion trap mass spectrometry (LC/ESI/MS/MS).

[073] To identify the DNA-PKcs *in vitro* T2609 phosphorylation site, purified DNA-PK was *in vitro* autophosphorylated under the conditions described above and analyzed by SDS PAGE. To identify the *in vivo* S2056 DNA-PKcs phosphorylation site, 10 L of HeLa S3 cells were irradiated with 25 Gy of ionizing radiation. Nuclear extracts made from the irradiated cells and immunoprecipitated, according to the method described in Example 3, with the 25-4 monoclonal antibody to DNA-PKcs (NeoMarkers, Lab Vision, Fremont, CA) and analyzed by SDS PAGE. All chemicals in this analysis were obtained from Fisher Scientific (Pittsburgh, PA).

[074] The coomassie blue-stained DNA-PKcs bands were excised from the gel and destained with 50 mM NH_4CO_3 in 50% methanol. Once destained, the gel slices were fixed overnight with 10% acetic acid and 50% methanol. The gel slices was then swelled with water for 2 hr and grounded to a fine powder in 10 μ L of 50 mM NH_4HCO_3 . Trypsin (Roche Diagnostics, Alameda, CA) was added and the samples were incubated at 37°C for 90 min.

The digested peptides were extracted from the gel with acetonitrile and concentrated by centrifugation with SpeedVac. A portion of the dried peptides was redissolved in 50 mM NH_4CO_3 for digestion with Asp-N protease (Roche Diagnostics, Alameda, CA) at 37°C for 90 min and dried. The dried tryptic and tryptic-Asp N peptides were dissolved in 10 μL of 50% acetonitrile for further processing. A portion of the sample was treated with calf intestine phosphatase (CIP) (New England Biolabs, Beverly, MA) in 50 mM NH_4CO_3 at 37°C for 90 min and dried by centrifugation in a SpeedVac. The dried peptides were redissolved in 2 μL of 50% acetonitrile for MALDI-TOF mass spectrometry.

[075] The CIP-treated and untreated peptides were analyzed in a Voyager DE

MALDI-TOF system from Perspective Biosystems. Most of the peaks in the spectrum of the tryptic digest could be easily assigned to unique peptides predicted from the protein sequence and to peptides formed by autolysis of trypsin. Peaks that could not be accounted for in this way were candidates for modified peptides, and those peptides having observed masses that were 80 Da (or multiples of 80 Da) higher than that calculated for a predicted tryptic peptide were tentatively assigned as phosphopeptides. This assignment was confirmed by the absence of these peaks from the MALDI/TOF spectrum of the same peptide mixture after treatment with CIP and the appearance of new peaks that are 80 Da (or multiples of 80 Da) lower in mass. Once the phosphorylated peptide was identified, it was then analyzed on an electrospray ion trap mass spectrometer (LCQ, Finnigan MAT, San Jose, CA) coupled on-line with a capillary HPLC (Magic 2002, Michrom BioResources, Auburn, CA) to identify the phosphorylation sites in the phosphorylated peptides.

[076] Referring now to Fig. 1, there is shown the mass spectra of one of the tryptic fragments. Fig. 1 is a mass spectra of sequencing of the *in vitro* phosphorylated DNA-PKcs peptide that was first identified by MALDI-TOF mass spectrometry. The plot shows a plot of relative intensity vs the mass-to-charge ratio (m/z) of the phosphorylated peptide having the sequence (shown in the Figure) from 2599 to 2619. Assignment of all the mass spectra peaks unequivocally identify T2609 as the site of phosphorylation. Thus, T2609 was unambiguously identified as a major site of autophosphorylation (Fig. 1).

[077] Referring now to Fig. 2, a second major *in vitro* and *in vivo* autophosphorylation site of DNA-PKcs, S2056, was identified by immunoprecipitating DNA-PKcs from nuclear extracts prepared from irradiated HeLa cells and analyzing by mass spectrometry (Fig. 2). DNA-PKcs from irradiated HeLa cells was purified by immunoprecipitation and digested with Asp-N protease. The peptide mixture was treated or mock treated with alkaline phosphatase and analyzed by MALD-TOF mass spectrometry.

Shown in Fig. 2 is the mass spectra of phosphatase-treated (bottom trace) and the mock treated (top trace) peptide mixture. The loss of the peak with the m/z of 3511 with phosphatase treatment, and the presence of the peak corresponding to the unphosphorylated peptide (m/z of 3433) allowed the inventors to positively assign a phosphorylation site to a sequence of DNA-Pkcs between amino acids 2044-2072. This peptide was then sequenced by tandem mass spectrometry (as in Fig 1) to unequivocally identify S2056 as the site of phosphorylation.

[078] Mass spectrometry identified the following phosphopeptide sequence, SEQ ID NO: 5, DFSTGVQSYSSQDPRPATGRFRRREQR, which corresponds to amino acids 2044 to 2072 of DNA-PKcs (S2056 is underlined).

EXAMPLE 2

DNA-PKcs Fragments and GST Fusion Proteins Containing Autophosphorylated Sites

[079] 20 bp oligomer primers were designed and ordered from Operon (Alameda, CA) using SEQ ID NO: 4 (the nucleotide sequence of DNA-PKcs, GenBank Accession Number: P78527) to create primers to amplify cDNA sequence that encodes the phosphorylation sites, T2609 and S2056. Designed DNA-PKcs cDNA fragments that cover the phosphorylation sites in DNA-PKcs found by mass spectrometry were PCR amplified from the full-length DNA-PKcs cDNA (isolated and described by several of the inventors in Kurimasa et al., *Mol Cell Biol* 19: 3877-3884, 1999) using the custom designed PCR primers under normal PCR thermal cycling conditions. The reactions were carried out using *pfu* DNA polymerase (Stratagene, La Jolla, CA) and GeneAmp 9600 thermocycler (Perkin Elmer). The amplified cDNA fragments were cloned in frame into GEX-KG vector (Guan & Dixon 1991 Analytical Biochem. 192:262-67) for fusion between domains of DNA-PKcs and GST.

[080] These clones were made to express the following residues of DNA-PKcs from the indicated corresponding cloned cDNA sequence: 1879-2182 cDNA (SEQ ID NO: 18), 1879-2267 cDNA (SEQ ID NO: 19), 2261-2700 cDNA (SEQ ID NO: 21), 2275-2702 cDNA (SEQ ID NO: 23), 2429-2702 cDNA (SEQ ID NO: 24), 2561-2700 cDNA (SEQ ID NO: 25), and 2600-2702 cDNA (SEQ ID NO: 26).

[081] Peptide fragments were expressed by the clones which encode the following peptides and correspond to the following residues of DNA-PKcs: 1879-2182 (SEQ ID NO:

6), 1879-2267 (SEQ ID NO: 7), 1879-2700 (SEQ ID NO: 8), 2261-2700 (SEQ ID NO: 9), 2500-2702 (SEQ ID NO: 10), 2275-2702 (SEQ ID NO: 11), 2429-2702 (SEQ ID NO: 12), 2561-2700 (SEQ ID NO: 13), and 2600-2702 (SEQ ID NO: 14).

[082] Several of these fragments were also made into glutathione-S-transferase (GST) fusion proteins. The PCR'd fragments were cut randomly and then fused with the GST protein using the commercially available GST fusion vector (Amersham Biosciences, Piscataway, NJ).

EXAMPLE 3

Preparation of Cellular Nuclear Extracts from Cells

[083] The preparation of nuclear extract from HeLa cells for the Examples that follow were made as generally described by Lees-Miller et al., *Mol Cell Biol* 10: 6472-6481, 1990 and is herein described. The cells were washed twice with cold PBS, collected, and spun at 2000g for 5 min. The cell pellet is washed once with 5 ml LSB and spun again. The pellet is resuspended in 1ml LSB and transfer to a centrifuge tube. (LSB (low salt): 10 mM Hepes pH7.5, 25 mM KCl, 10 mM NaCl, 1mM MgCl₂, 0.1mM EDTA).

[084] After spinning down again, the volume of the cell pellet is estimated, then resuspended in 1x Pack cell volume (PCV) of LSB (with 50 mM NaF, 1mM DTT, 0.5 mM PMSF, and other protease inhibitors), set in ice 5 min, and freezed in liquid N₂. Thaw, and spin immediately at 10,000g for 10 min. Dispose of Supernatant (S10, cytosol fraction).

[085] The pellet is again resuspended in 1x pack nuclear volume (PNV) of LSB with 0.5M NaCl and 10 mM MgCl₂ (500 mM NaCl, 10 mM MgCl₂, 50 mM NaF, 1mM DTT, 0.5 mM PMSF), set in ice for 10 min. For this step, LSB (0.5 M salt): 10 mM Hepes pH7.5, 25 mM KCl, 500 mM NaCl, 10mM MgCl₂, 0.1mM EDTA. The pellet is spun down at 40,000g for 20 min, supernatant (P10 nuclear fraction). The collected P10 nuclear extract is mixed 1:1 with 2X Laemmli buffer (80mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.1%BPB), then the sample is boiled at 100 °C for 3 min.

EXAMPLE 4

SDS Gels and Western Blots for DNA-PKcs

[086] Western blotting for the following Examples was performed as generally described by Chan et al., *Biochem Cell Biol* 74: 67-73, 1996 and herein described.

[087] **Preparation of 8% low bisacrylamide gels.** Resolution gel mix (10 ml): 3ml 30% acrylamide, 0.4ml 2% bisacrylamide, 2ml 1M Tris-HCl pH8.8, 3ml water, 100ul 10%

SDS, 100ul 10% APS, 8ul TEMED, allow to polymerize for at least 90 min. Stacking gel mix (5. ml): 3.4 ml water, 0.83 ml 30% acrylamide, 0.63 ml 1M Tris pH6.8, 50ul 10% SDS, 50ul 10% APS, 5ul TEMED, allow to polymerize for at least 30 min.

[088] **Gel Running.** Load the samples and run at 100V 1.5 to 2 hrs until BPB dye runs off. The electrophoresis running buffer is made as followed (per liter): 6 g Tris base, 28.8 g Glycine, 1 g SDS. Do not pH.

[089] **Transfer.** Remove gel from glass plates and place in 50ml electroblot, gently rock for 5-15 min. Electroblot (per liter): 5.8g Tris base, 2.93g glucine, 0.38 g SDS, 100ml methanol. For each gel to be blotted, prepare 2 squares of 3 mm filter paper cut to size of gel and place them in electroblot. Cut 1 square piece of nitrocellulose or PVDF membrane about the same size.

[090] Wet the PVDF membrane with Methanol, and equilibrate in electroblot for 5 min before use. For nitrocellulose, place directly in electroblot. Submerge blotting cassette and Scotchbrite pads in electroblot, assemble as follows: towards the black side of the cassette, scotchbrite (sponge), 3 mm filter paper, gel, nitrocellulose membrane, 3 mm filter paper, and then scotchbrite. Make sure that no air bubbles are trapped between the gel and the nitrocellulose membrane by smoothing out with gloved finger or rolling with a glass rod. Place assembly in transfer chamber with black side to black side; add the frozen cooling pack and fill to top with electroblot. Transfer at 100V (~250mA) for 1 hour on ice bath or 15V overnight at RT with gentle stirring of the electroblot.

[091] **Western Blot.** Place membrane in block solution (5% none fat milk powder in TTBS) for at least 60 min. TTBS: 10 mM Tris pH 8.0, 150 mM NaCl, 0.1% TWEEN-20.

[092] Incubate the blot with primary antibody for 1 to 2 hrs at RT, 1: 1000 dilution of purified DNA-PKcs antibodies in block solution. Wash blot with TTBS for 10 min, three times. Incubate blot with secondary antibody for 30 min at RT, 1: 5000 dilution of goat anti-rabbit HRP conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in TTBS.

[093] Wash blot with TTBS for 10 min, three times. Make up ECL solutions (Amersham Biosciences, Piscataway, NJ): 1ml of each A and B is sufficient for a blot. Immerse blot in ECL solution for 1 minute, remove from ECL solution, place in a sandwich of plastic wrap, expose blot to film in dark room for various times (5 sec to 10 min), and develop film.

EXAMPLE 5

Cellular Radiation Sensitivity and Defective DSB Repair in Cells Having T2609A and S2056A Mutant Proteins

[094] To investigate the biological significance of the T2609 and S2056 phosphorylation in relation to DNA repair, wild-type or mutant DNA-PKcs were tested for DNA DSB repair, and radiation survival. DNA-PKcs expression constructs were made and transfected into the DNA-PKcs-defective V3 CHO cell line (Kurimasa et al., *J Immunol.* 2000 Oct 1;165(7):3883-9). Stable V3 cell lines that were expressing wild-type DNA-PKcs (V3-F18), T2609A DNA-PKcs mutant proteins (V3-T2609A), S2056A DNA-PKcs mutant proteins (V3-S2056A) and S2056A/ T2609A DNA-PKcs double-mutant proteins (V3-S2056A/T2609A) were isolated and evaluated for DNA-PKcs protein expression levels, radiation sensitivity and DNA repair defects. The radiation sensitivity of these cell lines was examined by assaying for their colony forming ability after IR.

[095] The following was used to carry out site-directed mutagenesis and isolation of the mutant cell lines. The creation of the T2609A mutant (V3-T2609A) is herein described. The S2056A DNA-PKcs mutant (V3-S2056A) and S2056A/T2609A DNA-PKcs double-mutant (V3-S2056A/T2609A) were generated using the same methods but different primers.

[096] First, a 3kb Hind III fragment of DNA-PKcs cDNA covering T2609 was used as the template for generating the T2609A mutation of DNA-PKcs cDNA. Site-directed mutagenesis was performed using the Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA) and the forward (tccgatgtttgtggaggaccaggcctcccagggc) (SEQ ID NO: 27) and reverse (gccctgggaggcctggtcctccacaaacatcgga) (SEQ ID NO: 28) primers. The mutated DNA-PKcs cDNA fragment was assembled back into the full length DNA-PKcs cDNA as described in Kurimasa et al., *Mol Cell Biol* 19: 3877-3884, 1999. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air by using alpha-MEM medium supplemented with 10% fetal calf serum, 100 U of penicillin per ml, and 100 µg of streptomycin per ml. Transfection of the DNA-PKcs expression plasmid was performed with a calcium phosphate transfection system (Catalog no. 18306-019; Gibco-BRL, Gaithersburg, MD). For each 10⁶ cells in a 100-mm tissue culture dish, 10 µg of the DNA-PKcs expression vector and 10 µg of the pSV2neo or pPur plasmid were transfected.

[097] T2609A DNA-PKcs expression plasmid together with pSV2neo plasmid were transfected into the V3 cell line. Forty-eight hours after transfection, cells were replated on selection medium containing 400 µg/mL of G418 for 10 days. After 7 to 21 days of selection, individual colonies were isolated and further cultured.

[0098] Colony formation and FAR (Fraction of Activity Released) assays were performed as previously described (Kurimasa et al., *Mol Cell Biol* 19: 3877-3884, 1999).

[0099] Radiation survival assays generated survival curves for each cell line. These survival curves were obtained by measuring the colony-forming abilities of irradiated cell populations. Three hundred cells were plated on 60-mm plastic petri dishes and irradiated with ¹³⁷Cs γ rays at 2 h after plating at a rate of 2.2 Gy/min to achieve a cumulative dose of 1, 2, 3, or 5 Gy. After 7 to 14 days, cells were fixed and stained with 1% crystal violet in a 70% ethanol solution, colonies containing more than 20 cells were scored, and the mean value for triplicate culture dishes was determined. Cell survival was normalized to plating efficiency of untreated controls for each cell type.

[0100] Referring now to Fig. 3A, nuclear extracts were prepared as described in Example 3 from V3 cells transfected with vector alone (V3-JM), full length wild-type DNA-PKcs (V3-WT) or DNA-PKcs containing the T2609A point mutant (V3-T2609A1) were analyzed for DNA-PKcs protein expression levels (top panel). Nuclear extracts (P10) were prepared as described in Example 3. 20-60 μ g of each sample was analyzed by western blotting as described in Example 4. For analysis of the V3 cell lines, the DNA-cellulose pull-down method of Finnie et al. (*Proc Natl Acad Sci U S A* 92: 320-324, 1995) was used to first concentrate DNA-PKcs onto the cellulose and then subjected to SDS-PAGE for western blotting. Hamster Werner proteins were analyzed to demonstrate equal sample loading (bottom panel). DNA-PKcs protein levels in V3 (lane 1) is undetectable due to low abundance of DNA-PKcs RNA, whereas, V3-WT (lane 3) and V3-T2609A (lane 4) showed similar levels of protein expression (Fig. 3A, top panel). On the bottom, the expression of *wrn* (another DNA repair protein) was determined to show the equal loading of the samples.

[0101] Complementation of human DNA-PKcs in V3 cells restored radioresistance resulting in survival that is comparable to wild-type CHO cells (Fig. 3B). In Fig. 3B, the V3 cells complemented with the S2056A mutation DNA-PKcs (V3-S2056A), T2609A mutation DNA-PKcs (V3-T2609A) and S2056A/T2609A double mutation DNA-PKcs (V3-S2056A/T2609A) showed a radiation sensitivity phenotype that was more severe than the wild type. Although expression of the S2056A and T2609A mutant proteins improved the survival of the V3 cell line, the survival rates were significantly lower than what was observed for V3-WT. The dose of IR required for 10% survival of the V3-JM, V3-S2056A, V3-T2609A, V3-S2056A/T2609A, and V3-WT cell lines was 1.2 Gy, 1.9 Gy, 2.4 Gy, 2.1 Gy, and 5 Gy, respectively (Fig. 3B). Thus, the D₁₀ value is approximately four-fold higher

for V3-WT compared to the V3-JM non-complemented cells (that is 5 Gy/1.2 Gy), whereas resistance at the 10% survival level was increased by only two-fold in the V3-T2609A1 cells (5 Gy/2.4 Gy). However, the resistance at the 10% survival level is increased by about 2.5 times in the cell lines containing the S2056A mutation, thus showing the significance of phosphorylation of the S2056 site in radioresistance. At around 4 Gy, both the S2056A mutant and the double mutant drop below 1.0% survival rate, showing a greater radiosensitivity is caused by mutating the S2056 site than the T2609 site alone. At a dosage of 5 Gy, the T2609A mutant survival rate was less than 1% while the wild type cells having functional DNA-PKcs showed a 10% or greater survival rate. Therefore, phosphorylation of T2609 and S2056 are shown to be important for cell viability in response to ionizing radiation (IR).

[0102] The presence of additional DNA-PKcs phosphorylation sites may explain why the V3-T2609A1 cells showed only a roughly two-fold increase in radiation sensitivity (at 10 Gy, Fig. 3B). In response to DNA damage, phosphorylation of multiple sites may be required for proper DNA-PK function, and thus explaining why mutation of T2609 produced only a two-fold increase in radiation sensitivity, but the mutation of S2056 produced a greater increase to radiation sensitivity.

[0103] In Fig. 3C, T2609A mutation compromises DSB repair as shown by the FAR (fraction of activity released) Assay which is a DSB rejoining assay. The FAR Assay is used to analyze the mobility of genomic DNA in the polyacrylamide gel electrophoresis (PAGE) and to measure the presence of DNA DSBs. The FAR assay uses pulsed field gel electrophoresis to indirectly measure the intactness of DNA in cells after gentle lysis in agarose plugs by quantifying the amount of DNA released from the wells immediately after IR exposure as a function of dose or after a period of incubation to allow repair after a given dose (Story et al., *Int J Radiat Biol* 65: 523-528, 1994).

[0104] The V3-JM, V3-WT, V3-T2609A1 cell lines and the parental CHO cell line (AA8) were irradiated at the indicated dose and analyzed for the presence of DSBs by the FAR assay. DNA DSB repair activity following exposure to ionizing radiation was measured by two different methods: (i) rejoining kinetics, plotted as a function of time course after irradiation; and (ii) measure of residual DNA DSB lesions following exposure and recovery to three doses (0, 20, and 40 Gy) of ¹³⁷Cs γ rays. Exposures consisted of a dose rate of about 4 Gy/min on ice. Immediately following irradiation, the cold medium was replaced with medium that had been warmed to 37°C and the cells were placed in a 37°C tissue culture incubator for 4 h to allow for DNA DSB repair. The cells were then trypsinized on ice,

washed, suspended in agarose plugs, lysed, and electrophoresed. Residual DNA DSB lesions were determined by CHEF pulsed-field gel electrophoresis combined with a storage phosphorimaging system. Rejoined lesions were defined as the fraction of DNA that had regained sizes large enough to prevent migration during electrophoresis (DNA retained) and measured by comparing the intensity of fluorescence of the DNA fraction retained in the agarose well.

[0105] Referring now to Fig. 3C, there is a graph showing the results of the FAR assay utilized to evaluate the ability of each of the three cell lines to rejoin DSBs induced by IR. The graph plots the percentage of DNA retained in the well (which shows DSB rejoining capacity) versus total radiation dosage. The V3-WT and the parental AA8 CHO cell lines showed comparable DSB rejoining capacities (Fig. 3C, open (80% retained) and closed circles (85% retained), respectively) and . In contrast, the V3-JM and the V3-T2609A cells were significantly more defective in the rejoining of DNA DSBs at 4 hrs after irradiation (only 70% of DNA retained in well), consistent with previous observations (Kurimasa et al., *Mol Cell Biol* 19: 3877-3884, 1999) and with the hypothesis that DNA-PKcs plays an important role in repair of DSBs. Together, these results show that phosphorylation of DNA-PKcs at T2609 is important for rejoining of DSBs and for cell survival in response to DNA damage caused by IR.

EXAMPLE 6

Generation and Specificity of Phosphospecific Polyclonal Antibodies to pT2609 Site and pS2056 Site

[0106] To study the *in vivo* phosphorylation status of DNA-PKcs at T2609 and S2056, phosphospecific antibodies were generated. The phosphospecific antibodies, pT2609Ab, recognizes phosphorylated T2609, and pS2056Ab recognizes phosphorylated S2056. pT2609 polyclonal antibodies were prepared by immunizing New Zealand white rabbits with a KLH-conjugated phosphopeptide, N'-TPMFVET[PO³]QASQGT-C' (SEQ ID NO: 1). pS2056 polyclonal antibodies were prepared by immunizing New Zealand white rabbits with KLH-conjugated phosphopeptide, N'-QSYSS[PO³]QDPRPAC-C' (SEQ ID NO: 2).

[0107] **KLH-Conjugated Phosphopeptide.** To create the KLH-conjugated phosphopeptide (SEQ ID NO: 1 and 2), the phosphopeptide was made by conventional oligonucleotide synthesis means by AgBio, Inc. (Fremont, CA). 10 mg of the phosphopeptide was dissolved in 0.05 mL DMSO first, then 1 mL PBS (PBS: 0.1 Phosphate

buffer + 0.15 M NaCl, pH 7.3) was added and mixed. Activated KLH (10 mg Soluble keyhole limpet hemacyanin) (Calbiochem #374817, EMD Biosciences, San Diego, CA) was mixed with the dissolved peptide and the mixture was incubated at 4 °C with gentle rotation for overnight or room temperature (RT) for 3 hours. At the end of the first incubation, 10 mg
5 cysteine (L-cysteine HCl, Sigma, St. Louis, MO) in 2 mL PBS was added to the mixture, then vortexed briefly.

[0108] The phosphopeptides were conjugated to KLH by cross-linker Sulfo-SMCC (Pierce Biotechnology, Inc., Rockford, IL) which forms a disulfate bridge with the cysteine residues placed at the C' terminal of the synthesized peptides and cysteine residues on KLH.

10 The mixture was then incubated with rotation at RT for 2 hours to block unreacted SMCC. Dialysis against 2L PBS was done with at least 2 buffer changes. Dialysis may proceed overnight. In place of dialysis, a SEPHADEX G-25 column (Amersham Biosciences, Piscataway, NJ) may be used again to desalting.

[0109] The peptide/KLH conjugate solution was collected in dialysis bag or in
15 fractions (in case of G-25 gel) and 18 mg NaCl to each mL of the solution was dissolved to give the solution additional 0.3 M NaCl. The solution was centrifuged if particulates or precipitates observed. The protein concentration of the clear conjugate solution was measured by using $A_{280\text{nm}}$ and a coefficient $1.4 = 1 \text{ mg/mL}$. The conjugate solution was then diluted to 1 mg/mL and aliquoted 1 mL of the peptide-KLH conjugate solution into tubes.
20 Each tube contained 1 mg of the conjugate and was sufficient for one immunization dose for two rabbits. The tubes were stored at -20 °C until use.

[0110] The polyclonal antibodies were made by immunizing New Zealand white rabbits with the above KLH-conjugated phosphopeptide, (SEQ ID NO: 1 and 2) using standard methods well known in the art by AgBio, Inc (Fremont, CA). Crude rabbit serum
25 was collected from the immunized rabbits. The KLH-conjugated phospho peptides were then mixed with adjuvant and were injected into a rabbit through intradermal injection to elicit immunogenic response. After repeated injection to boost the immunogenic response, samples of serum were collected and tested by ELISA assay (to determine the titer of the antibodies) until the titer reached to the peak. The antibodies were then harvested.

30 [0111] The phosphospecific antibodies were affinity purified through a phosphopeptide-conjugated Sepharose CL-4B column. SEQ ID NO: 1 was made as an unphosphorylated peptide, N'-PMFVETQASQGTC-C' which corresponds to the T2609 site

unphosphorylated. SEQ ID NO: 2 was made as an unphosphorylated peptide, N'-QSYSYSSQDPRPAC-C', to correspond to the S2056 site unphosphorylated.

[0112] The following protocol was used to affinity purify the pT2609 and pS2056 rabbit polyclonal antibodies. Two columns are needed. One column uses an unphosphorylated version of the phosphopeptides used to immunize the rabbits. A second column uses the phosphopeptides. Eluted IgGs are passed through the first unphosphorylated peptide column to deplete any IgGs that are not specific to pT2609 or pS2056 and then the flow-through is then passed through the second phosphopeptide column to affinity purify the polyclonal antibodies specific for pT2609 and pS2056.

[0113] To prepare the columns, dissolve the appropriate peptide (1 mg/per ml) in coupling buffer: 50 mM Tris pH 8.5, 5 mM EDTA. Pack 5 ml SulfoLink Coupling Gel (Pierce Biotechnology, Rockford, IL) in 10 ml disposable polystyrene column (Pierce Biotechnology), equilibrate the column with 6 column volumes of coupling buffer. Place the bottom cap to the column, and add 5 ml peptide solution (5 mg) to the column. Place top cap, and mix the column at RT for 15 minutes with gentle rotation. Set for 30 minutes without mixing. Drain buffer, wash column with 3 column volumes of coupling buffer. Place the bottom cap to the column, and add 5 ml 50 mM cysteine to the column. Place top cap, and mix the column at RT for 15 minutes with gentle rotation. Set for 30 minutes without mixing. Drain buffer, wash column with 16 column volumes of 1 M NaCl.

[0114] For affinity purification, wash both non-phospho and phosphospecific peptide columns with 5 column volumes of PBS. Load 15 to 30 ml crude rabbit serum onto non-phosphopeptide column in RT. Collect flow-through. This step is to remove none specific antibodies. Load the flow-through onto phosphospecific peptide column with PBS. Wash with 10 column volumes of PBS with 0.5 M NaCl. Elute with 3 column volumes of 0.1 M glycine pH2.5, collect 1 ml fraction and neutralize the pH with 50 μ l 1M Tris pH 8.0, and check protein concentration by Bradford assay (Bio-Rad, Richmond, CA) and freeze in -20°C.

[0115] In Fig. 4A, GST fusion proteins, having fragments spanning amino acids 2500-2700 (SEQ ID NO: 10) fused to GST, were made according to Example 2. The fragments contained either the wild-type DNA-PKcs sequence or the T2609A point mutation. The GST fusion proteins were *in vitro* phosphorylated with purified DNA-PK as described in Example 2 and analyzed by Western blot according to Example 5. The wild-type DNA-PKcs sequence and the T2609A point mutation were probed with the pT2609pAb (top panel) and anti-GST (bottom panel) to show equal loading. The lack of any signal detected for T2609A

by the pT2609pAb of the invention in the Western blot shows that T2609 in DNA-PKcs is phosphorylated and that the pT2609pAb is specific for the phosphorylated T2609 site.

[0116] Referring now to Fig. 4B, the Western blot shows that pT2609pAb is specific to phosphorylated T2609 and does not recognize unphosphorylated DNA-PKcs. In Fig. 4B, affinity-purified pT2609 polyclonal antibody was used in immunoblotting with mock or autophosphorylated DNA-PKcs, lanes 1 and 2, respectively, and in the presence of excess unphosphorylated DNA-PKcs at the indicated molar excess ratios. Immunoblotting with unphosphorylated DNA-PKcs at 100 fold molar excess (relative to the phosphorylated DNA-PKcs) did not produce a detectable signal. Western blotting with pT2609pAb (top panel) and 25-4 DNA-PKcs monoclonal antibody (bottom panel) with mock or autophosphorylated DNA-PKcs (lanes 1 and 2, respectively) and with purified, unphosphorylated DNA-PKcs (lanes 3 to 9) at the indicated molar ratios relative to the amount of protein in lanes 1 and 2.

[0117] Referring now to Fig. 4C, a similar experiment shows that affinity purified pS2056Ab is specific to phosphorylated S2056. V3 (DNA-PKcs deficient CHO cells) complemented with either wild type human DNA-PKcs cDNA (V3-F18) or kinase dead mutant (V3-KA4) were subjected to mock or 10 Gy of ionizing radiation. DNA-PKcs protein was immunoprecipitated from nuclear extracts by 25-4 DNA-PKcs monoclonal antibody, and western blotted with the generated pS2056 rabbit polyclonal antibody (bottom panel). The blot was stripped and reprobed with the 25-4 DNA-PKcs monoclonal antibody (top panel). S2056 phosphorylation was diminished in V3-KA4 (kinase dead mutant) as compared to that of V3-F18 (wild type DNA-PKcs) indicating that DNA-PKcs autophosphorylation is responsible for IR-induced S2056 phosphorylation.

EXAMPLE 7

Localization of pT2609 Antibody to Site of DNA Double-Strand Breaks

[0118] In response to DNA damage, many DNA repair proteins form nuclear foci, presumably, the site of the DNA DSBs (Rogakou, *J Cell Biol* 1999 Sep 6; 146(5):905-16; Maser et al., *Mol Cell Biol*. 1997 Oct; 17(10):6087-96). To determine the status of DNA-PKcs in response to DNA damage, DNA-PKcs was examined by immunofluorescence microscopy. Immunostaining with a monoclonal antibody to DNA-PKcs produced strong signal throughout the nucleus, in both unirradiated and irradiated cells (not shown).

[0119] Fluorescent immunostaining showed that pT2609pAb produce foci only in irradiated but not unirradiated primary human skin fibroblasts (HSF). Immunofluorescence was performed as previously described in (Burma et al. 2001, *J Biol Chem* 276: 42462-

42467). In contrast, immunostaining with 25-4 (a commercial monoclonal antibody to DNA-PKcs from Neomarkers, Lab Vision Corp, Fremont, CA) produced strong signal throughout the nucleus, in both unirradiated and irradiated HSFs, because DNA-PKcs is a very abundant nuclear protein.

5 [0120] Because DNA-PKcs is a very abundant nuclear protein, it is not possible to distinguish any foci in response to DNA damage with the monoclonal antibody. However, with the pT2609 polyclonal antibody, foci can be clearly detected in response to IR treatment. The number and size of foci varied with the dose of IR and reaches a maximum with 10 Gy (data not shown). In addition, the kinetics of the pT2609pAb foci formation is similar to
10 what was observed with the time-course western results in Fig. 5B.

[0121] To further confirm the localization of T2609 phosphorylation at DSB sites, we examined the colocalization of rabbit pT2609 polyclonal antibody foci with the p53 binding protein (53BP1) which has been previously shown to bind to the site of DNA DSBs (Rappold et al., *J Cell Biol.*, Apr 30;153(3):613-20 2001). Co-immunostaining with a 53BP1
15 monoclonal antibody (Rappold et al., *J Cell Biol.*, Apr 30;153(3):613-20 2001) and the pT2609Ab in unirradiated HSF cells did not produce any discernable foci above the background signal of the nucleus. 53BP1 monoclonal antibody was provide by Dr. Junjie Chen (Mayo Clinic, Rochester, MN). However, cells that were irradiated resulted in very discrete 53BP1 and pT2609pAb foci. Moreover, the 53BP1 and pT2609pAb foci co-
20 localized with each other. Thus by virtue of co-localization with 53BP1 foci in response to DNA damage, it was demonstrated that the pT2609pAb binds and becomes activated at the site of DNA DSB *in vivo*.

EXAMPLE 8

25 T2609 is Phosphorylated in Response to Irradiation *in vivo*.

[0122] Referring now to Fig. 5A, 50 μ g of HeLa nuclear extracts made from unirradiated (lane 3) or cells irradiated with 25 Gy and harvested after a 30 min recovery period, were analyzed by western blotting with pT2609pAb (upper panel) or 25-4 monoclonal antibody (bottom panel). As shown in Fig. 5A, phosphorylation of T2609 is
30 DNA damage inducible and was detected as early as 10 minutes and reached a maximum at approximately 30 minutes after treatment. T2609 is phosphorylated up to 4 hours after IR treatment detectable, after which the phosphorylation of T2609 is not detectable (Fig. 5A). Purified DNA-PKcs was mock (lane 1, control) or autophosphorylated (lane 2) and analyzed by western blotting using the pT2609 polyclonal antibody of the invention. HeLa cells were

either mock treated or irradiated with 10 Gy and allowed to recover for the indicated times. Nuclear extracts were western blotted with pT2609pAb (top panel) and then blots were stripped and reprobed with the 25-4 DNA-PKcs monoclonal antibody (Neomarkers, Lab Vision Corp, Fremont, CA) (bottom panel). Since phosphorylation of T2609 can be detected
5 as early as 10 minutes after IR, this suggests that phosphorylation of DNA-PKcs is an early event in response to DNA damage and is consistent with the hypothesis that DNA-PK is required for the early and rapid phase of the "biphasic" model of DSB repair. This biphasic model was previously described in DiBiase et al., *Cancer Res* 2000 Mar 1;60(5):1245-53.

[0123] Phosphorylation of T2609 is also dose dependent, and can be induced with 2
10 Gy of IR and reaches a maximum or saturation with 10 Gy of IR. As shown in Fig. 5B, HeLa cells were irradiated with the indicated dose of IR and allowed to recover for 30 min. Nuclear extracts were first analyzed by western blot with pT2609pAb (top panel) and then with 25-4 monoclonal to show equal loading (bottom panel). Since phosphorylation of T2609 can be observed with a dose as low as 2 Gy, these results suggest that phosphorylation
15 of DNA-PKcs is very sensitive to the presence of DSBs in the genome. The inventors have also observed the same phosphorylation of T2609 in response to IR in a lymphoblastoid cell line (Jurkat), a glioma cell line (M059K) and in primary human fibroblasts (data not shown), and thus this event appears to be a general phenomenon that is not cell-type specific.

[0124] Phosphorylation at T2609 in response to DNA damage was further confirmed
20 by immunoprecipitation with the pT2609pAb. As shown in Fig. 5C, pT2609 polyclonal antibody was used to immunoprecipitate DNA-PKcs from 500 μ g of unirradiated HeLa nuclear extract (lane 1) or extracts made from HeLa cells irradiated with 25 Gy and harvested after 30 min recovery period. DNA-PKcs was immunoprecipitated with the pT2609pAb only in the nuclear extracts prepared from irradiated cells but not from that of untreated cells, thus
25 showing that the pT2609pAb is specific for phosphorylated DNA-PKcs.

[0125] In Fig. 5D, phosphorylation of T2609 in response to DNA damage can be inhibited with wortmannin treatment and is inducible in A-T cells indicating that DNA-PKcs autophosphorylation is responsible for T2609 phosphorylation *in vivo*. The activity of PI-3 kinase family members, including DNA damage responsible DNA-PK and ATM, are
30 sensitive to low dose of wortmannin (Sarkaria et al., *Cancer Res.* 1998 Oct 1;58(19):4375-82). Since phosphorylation of T2609 is through an autophosphorylation mechanism, then one would expect phosphorylation to be sensitive to the effects of wortmannin. Treatment of

HeLa cells with 20 μ m wortmannin, resulted in a decrease in the detectable levels of phosphorylated T2609 (Fig. 5D).

[0126] To determine whether the ATM (ataxia-telangiectasia-mutated) kinase may phosphorylate T2609 in response to IR, ATM deficient lymphoblastoid cell line (L3) and ATM positive cell line (BT) were treated with ionizing radiation and probed with pT2609pAb. In response to IR, phosphorylation of T2609 was observed in both the ATM wild-type (BT) and mutant (L3) cell lines, thus phosphorylation of DNA-PKcs at T2609 is ATM-independent.

EXAMPLE 9

Phosphorylation of S2056 *in vivo*

[0127] The observed S2056 phosphorylation *in vivo* data is similar to that of T2609. The experiments described in the previous Example were performed using p2056 polyclonal antibody (pS2056pAb) to observe the phosphorylation of S2056 *in vivo*. Referring now to Fig. 6A, phosphorylation of S2056 induced by irradiation *in vivo* was observed in HeLa cells (lane 2), wild type fibroblasts (lane 4), as well as ATM (ataxia-telangiectasia-mutated) deficient fibroblasts (lanes 6 and 7). S2056 phosphorylation in response to IR in ATM deficient fibroblasts also indicates that DNA-PKcs autophosphorylation is likely responsible for S2056 phosphorylation *in vivo*. The gel in Fig. 6A shows that the pS2056pAb detects phosphorylated DNA-PKcs only in irradiated cells (lanes 2, 4, 6 and 7) but not unirradiated cells (lanes 1, 3, 5 and 8). In addition, fluorescent immunostaining with pS2056 antibody show that pS2056pAb detects only phosphorylated DNA-PKcs and is localized at DSB sites (nuclear foci) only in irradiated but not unirradiated cells (data not shown).

[0128] Referring now to Fig. 6B, a main difference between T2609 and S2056 phosphorylation is that prolonged phosphorylation of S2056 can be detected upon DNA damage. S2056 phosphorylation can be detected six to eight hours after IR whereas T2609 phosphorylation is diminished after about 4 hours indicating the phosphorylation at T2609 and S2056 may have overlapping and distinct functions.

EXAMPLE 10

Generating pT2609 and pS2056 Monoclonal Antibodies

[0129] Specific mouse monoclonal antibodies against pT2609 and pS2056 were prepared using immunogens disclosed herein. Protocols for immunization and construction

of hybridomes may be found in U.S. Pat. No. 4,455,296 to Hansen et al and U.S. Pat. No. 4,364,933 to Kong et al and are hereby incorporated by reference. The screening process, is as described in Example 6. The cell lines and monoclonal antibodies recognizing only the phosphopeptides but not non-phosphopeptides are selected. Therefore, there is no need of affinity purification of Example 6 for the phospho specific mouse monoclonal antibodies. Monoclonal antibodies to phosphorylated T2609 (pT2609mAb) and phosphorylated S2056 (pS2056mAb) were generated according to the protocol herein described.

[0130] Female BALB/c mice were subcutaneously injected with 100 μ g/mouse into one spot with the phosphopeptide once a month for 4 months. The phosphopeptide was added to incomplete Freund's adjuvant. Testbleeds from mice's tail were drawn once a month after sterilizing mice skin with 70% alcohol. After 4 months of immunization, mice were given a final booster injection 4 days before doing fusion. The phosphoprotein was injected without adjuvant. The polyclonal mouse serum was collected from the hearts. The mice were sacrificed and the spleens and NS-1 myeloma cells were harvested.

[0131] The fusion of the harvested mouse NS-1 cells and spleen cells (B lymphocyte cells) was carried out according to the following method. Prepare hypoxanthine-aminopterin-thymidine (HAT, Sigma) medium (300 ml 15% RPMI medium + HTA) two days before. Defrost NS-1 cells (ATCC, Manassus, VA) and cultured in 15% RPMI medium (Gibco BRL, Gaithersburg, MD) (with 15% Fetal Bovine Serum obtained from Hyclone (Logan, UT)). Prewarm 2 ml 50% PEG, 5 and 15 ml RPMI, and 15% RPMI-HAT medium at 37°C.

[0132] Rinse the spleen in 5 mL RPMI in 60 mm petri dishes five times. Collect the spleen cells and put into 15 ml centrifuge tubes (4°C). Pass the spleen cells through 25-gauge needles three times and 21-gauge needles three times. Spin at 4°C, 1800 rpm, 5 minutes. Collect NS-1 cells (about 200 ml culture) and put into 50 ml centrifuge tubes.

[0133] Spin at 4°C, 1400 rpm, 5 minutes. Resuspend spleen cells with 5 ml RPMI medium and stand for 2 minutes. Transfer the supernatant of spleen cells into a new 50 ml tube and repeat spin at 4°C, 1400 rpm, 5 minutes and resuspend spleen cells with 5 ml RPMI medium and stand for 2 minutes. Re-suspend the NS-1 cells in 15 ml RPMI and transfer into one 50 ml tube. Spin NS-1 cells, resuspend pellets and spin, and then resuspend the pellets with 20 ml RPMI medium. Spin spleen cells, 4°C, 1800 rpm, 5 min, resuspend the pellets with 4 ml RPMI medium. Count cell numbers in 2 μ L spleen cells in 88 μ l ammonium chloride (incubate 5 min, lysis RBC) and 10 μ l trypan blue (before counting). Count number of cells in 5 μ l NS-1 cells, 40 μ l PBS and 5 μ l trypan blue.

[0134] Keep some spleen (5×10^6) and NS-1 (1×10^6) cells as control. Transfer optimum amount of NS-1 cells into spleen cell tube. The optimum amount is 1:5 = NS-1 cells:Spleen cells. Spin at 4°C , 1800 rpm, 5 min and completely remove the supernatant. Drop by drop over 1 min period, add 1 ml pre-warmed 50% PEG with continually shaking the tube, shaking 30 sec, stand for 1 min (37°C). Drop by drop over 5 min period, add 5 ml RPMI medium to the fusion mixture while gently agitating (37°C). Immediately add 15 ml RPMI medium over a 1 min period. Incubate the fusion mixture in 37°C water bath for 5 min. Spin down the cell pellets at 25°C , 1800 rpm, 5 min. Re-suspend the cell pellets with 15% RPMI-HAT medium (1×10^6 spleen cells/ml). Use normal mouse splenocytes as feeder cells (seeding before cell fusion). Seed the cell suspension in 96 well plate ($200 \mu\text{l}/\text{well}$), culture two weeks. Screen positive clones by dot blot assay.

[0135] The present examples, methods, procedures, treatments, specific compounds and sequences are meant to exemplify and illustrate the invention and should in no way be seen as limiting the scope of the invention. Any patents or publications mentioned in this specification are indicative of levels of those skilled in the art to which the patent pertains and are hereby incorporated by reference to the same extent as if each was specifically and individually incorporated by reference.

CLAIMS

What is claimed is:

- 5 1. An antibody which specifically binds to an epitope defined by at least a ten amino acid sequence from human DNA-PKcs, said sequence comprising:
a phosphorylated threonine at position T2609, wherein:
said antibody does not bind when T2609 is not phosphorylated.
- 10 2. The antibody of claim 1, wherein:
said antibody is a monoclonal antibody.
3. The antibody of claim 2, wherein:
said monoclonal antibody is a human monoclonal antibody
- 15 4. The antibody of claim 1, wherein:
said antibody is pT2609mAb.
5. An antibody which specifically binds to an epitope defined by at least a ten amino acid sequence from human DNA-PKcs, said sequence comprising:
20 a phosphorylated serine at position S2056, wherein:
said antibody does not bind when S2056 is not phosphorylated.
6. The antibody of claim 5, wherein:
25 said antibody is a monoclonal antibody.
7. The antibody of claim 6, wherein:
said monoclonal antibody is a human monoclonal antibody.
- 30 8. The antibody of claim 5, wherein:
said antibody is pS2056mAb.

9. A method for determining the ability of a test compound to block phosphorylation of human DNA-PKcs, comprising:
providing a sample containing a DNA-PKcs peptide fragment capable of being phosphorylated,
5 combining said test compound with said sample,
inducing phosphorylation of the DNA-PKcs peptide fragment, and
measuring the resulting phosphorylation of said DNA-PKcs peptide fragment at T2609 and/or S2056 in the presence of the test compound.
- 10 10. The method of claim 9, wherein:
said measuring step is carried out by measuring the binding of an antibody which specifically binds to an epitope comprising either or both of (a) a phosphorylated serine at position S2056 in human DNA-PKcs or (b) a phosphorylated threonine at position T2609 in human DNA-PKcs.
- 15 11. The method of either of claims 9 or 10, wherein:
said DNA-PKcs peptide fragment is an isolated peptide having less than 1000 amino acids, and comprising: SEQ ID NO: 4, SEQ ID NO: 5, or sequences having at least 90% homology thereto.
- 20 12. The method of either of claims 9 or 10, wherein:
said DNA-PKcs peptide fragment is selected from the group consisting of SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13 and SEQ ID NO: 14.
- 25 13. The method of either of claims 9 or 10, wherein:
said test compound is selected from the group consisting of: wortmannin, substituted or unsubstituted imidazoles, substituted or unsubstituted pyrazoles, substituted or unsubstituted fluoranthenes, substituted or unsubstituted thiazoles, substituted or unsubstituted quinolinones, substituted or unsubstituted phthalazinones, and derivatives thereof.
- 30

14. An isolated peptide having less than 1000 amino acids, comprising:
SEQ ID NO: 4, SEQ ID NO: 5, or sequences having at least 90% homology thereto.

5

15. The isolated peptide of claim 14, wherein:
T2609 and/or S2056 are replaced by an amino acid selected from the group consisting
of: Valine, Alanine, Glycine, and Leucine.

- 10 16. An isolated polynucleotide encoding the peptide of either claims 14 or 15.

17. The isolated polynucleotide of claim 16, having a sequence selected from the group
consisting of: SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19,
SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO:
15 24, SEQ ID NO: 25, and SEQ ID NO: 26.

18. A method of measuring radiosensitivity of cells in a subject, comprising:
(a) providing a cell sample from said subject, said sample containing subject's DNA-
PKcs,
20 (b) combining said sample with a labelled antibody which binds to phosphorylated
residue T2609 or phosphorylated residue S2056 but not the unphosphorylated
residues,
(c) removing any unbound antibody from the sample, and
(d) measuring the degree of phosphorylation of the DNA-PKcs by determining the
25 extent of binding of the antibody to the DNA-PKcs,
whereby the degree of antibody binding to DNA-PKcs correlates to the degree of
phosphorylation, a higher degree of phosphorylation indicating less radiation
sensitivity.

30

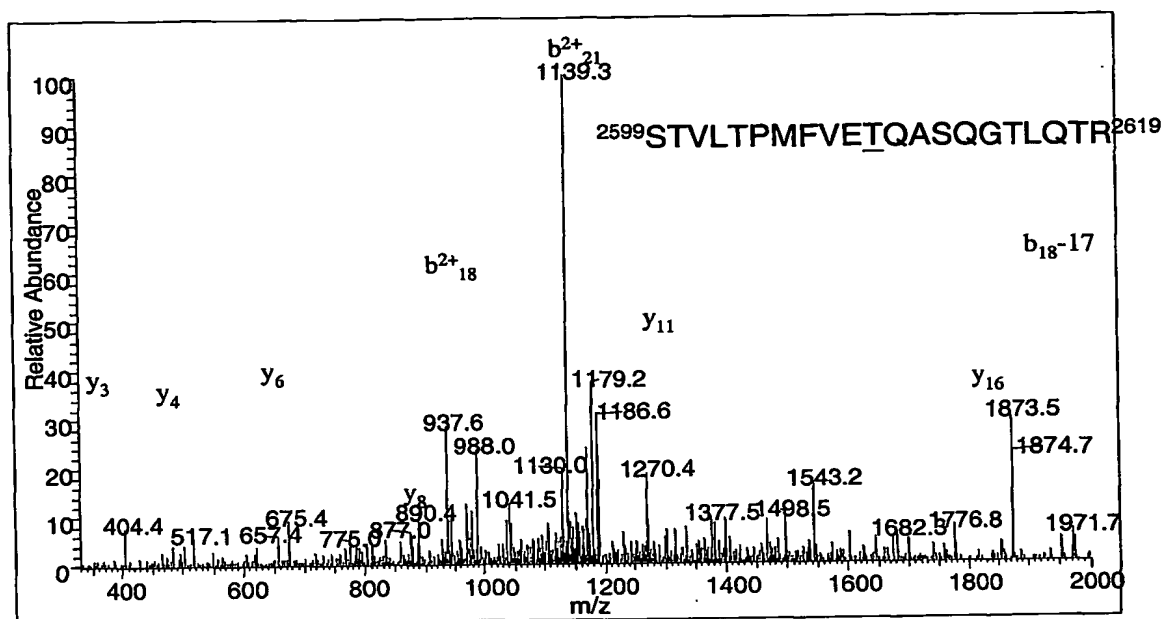
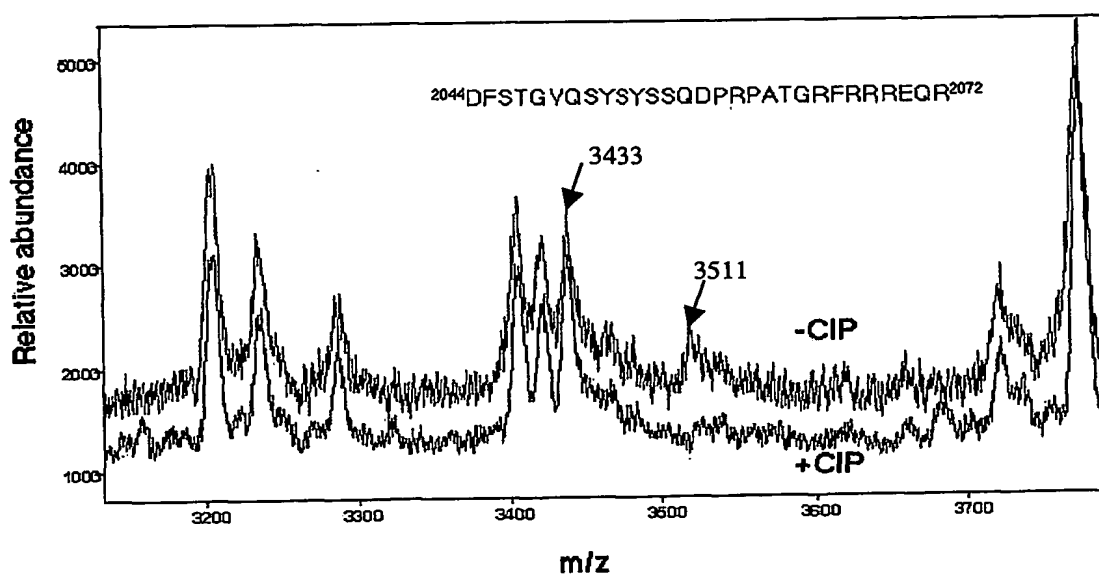
Fig. 1**Fig. 2**

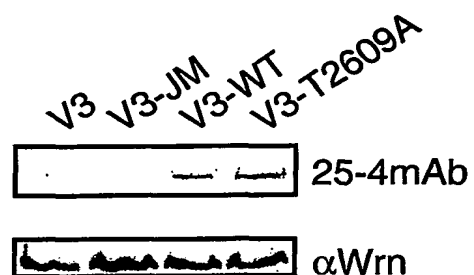
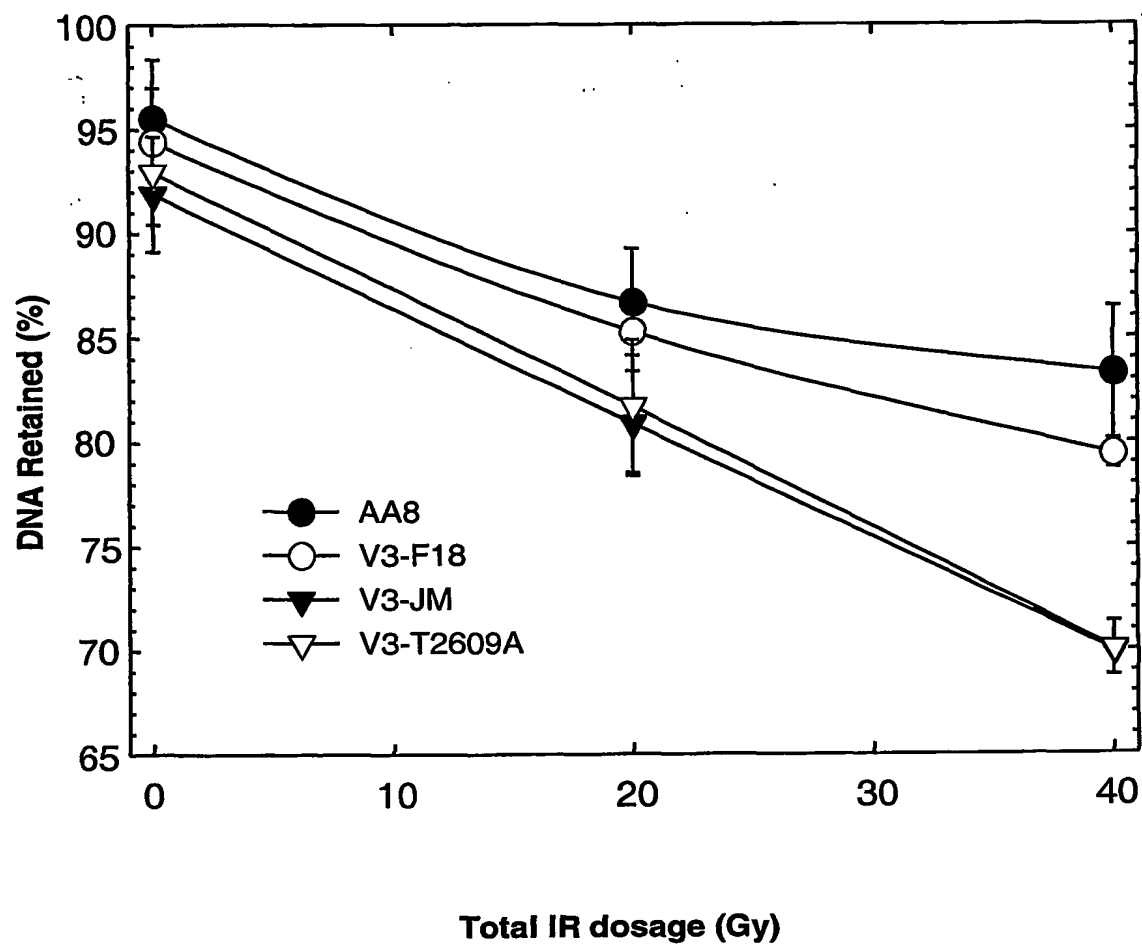
Fig. 3A**Fig. 3C**

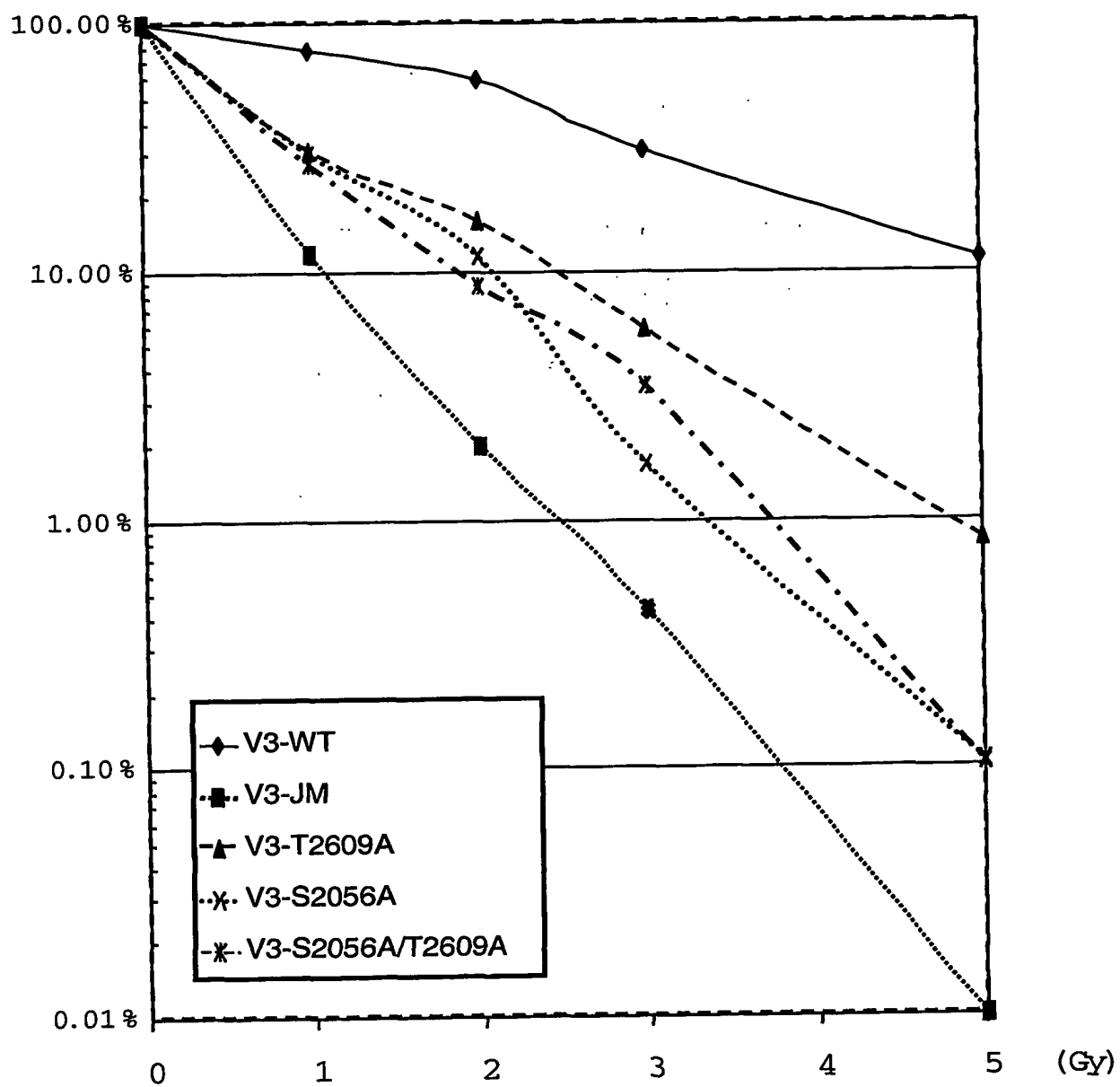
Fig. 3B

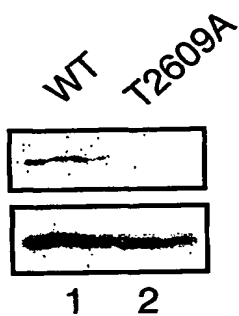
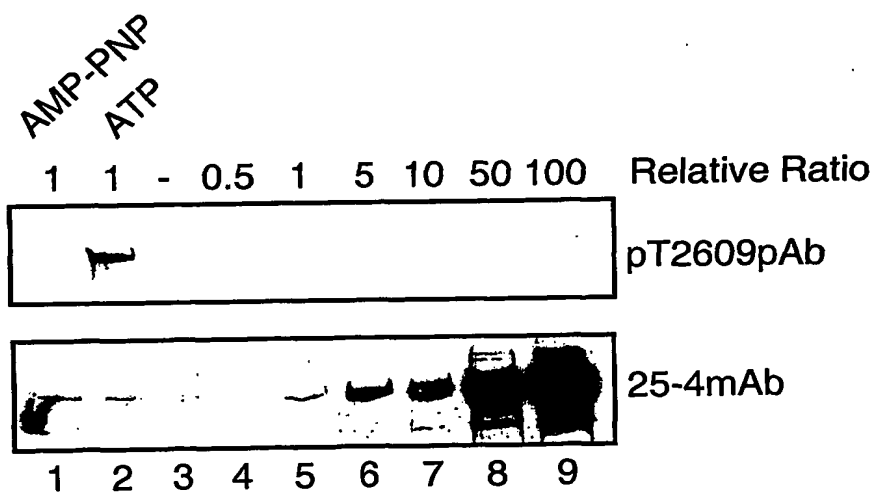
Fig. 4A**Fig. 4B**

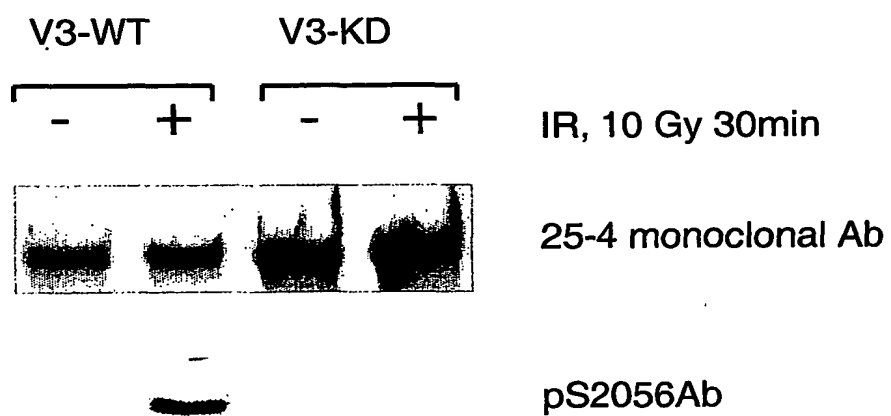
Fig. 4C

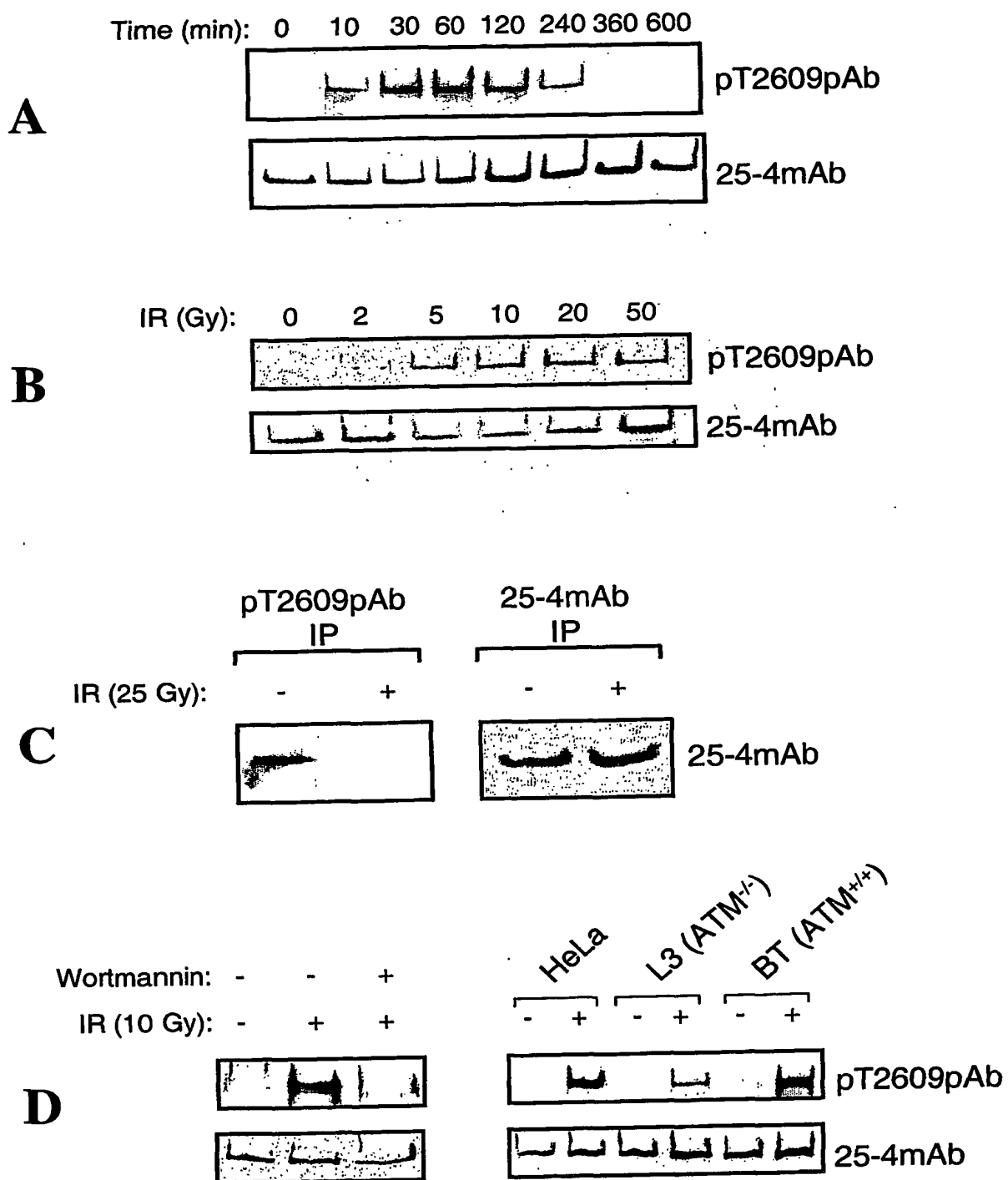
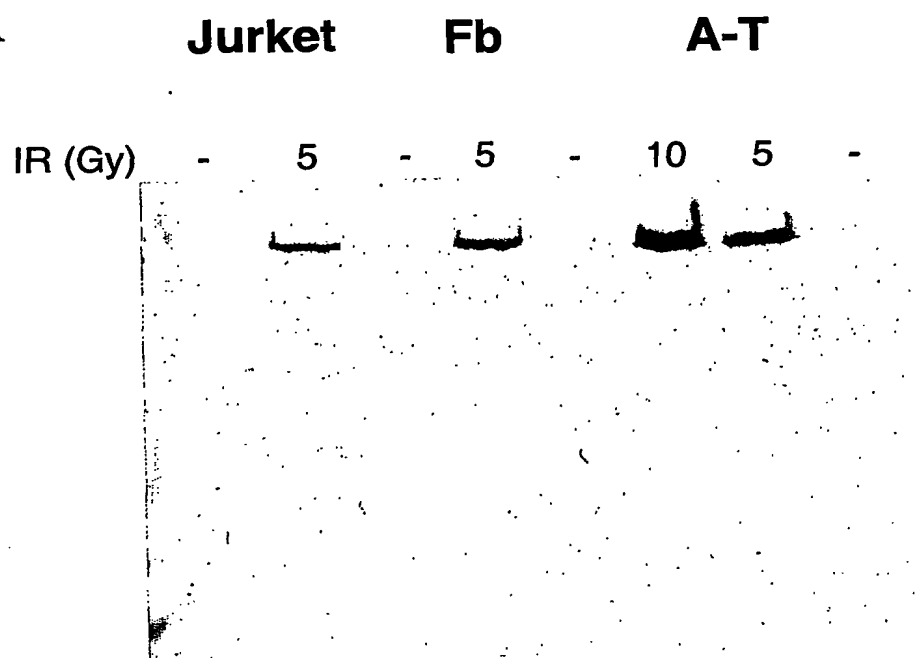
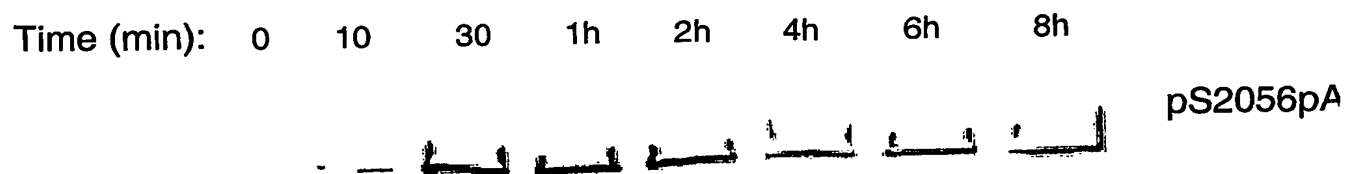
Fig. 5

Fig. 6**A****B**

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Chen, David J

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and Antibodies Thereto

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<141> 2003-04-21

<150> US 60/375,094

<151> 2002-04-22

<160> 28

<170> PatentIn version 3.1

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<213> Artificial Sequence

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<222> (1)..(14)

<223> HUMAN GENETIC ORIGIN

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<221> MOD_RES

<222> (7)..(7)

<223> PHOSPHORYLATION at T2609

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<210> 2

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<212> PRT

<213> Artificial Sequence

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<222> (1)..(14)

<223> HUMAN GENETIC ORIGIN

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<223> PHOSPHORYLATION at S2056

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<210> 3

<211> 4128

<212> PRT

<213> Homo sapiens

<400> 3

Met Ala Gly Ser Gly Ala Gly Val Arg Cys Ser Leu Leu Arg Leu Gln
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Glu Thr Leu Ser Ala Ala Asp Arg Cys Gly Ala Ala Leu Ala Gly His
 20 25 30

Gln Leu Ile Arg Gly Leu Gly Gln Glu Cys Val Leu Ser Ser Ser Pro
 35 40 45

Ala Val Leu Ala Leu Gln Thr Ser Leu Val Phe Ser Arg Asp Phe Gly
 50 55 60

Leu Leu Val Phe Val Arg Lys Ser Leu Asn Ser Ile Glu Phe Arg Glu
 65 70 75 80

Cys Arg Glu Glu Ile Leu Lys Phe Leu Cys Ile Phe Leu Glu Lys Met
 85 90 95

Gly Gln Lys Ile Ala Pro Tyr Ser Val Glu Ile Lys Asn Thr Cys Thr
 100 105 110

Ser Val Tyr Thr Lys Asp Arg Ala Ala Lys Cys Lys Ile Pro Ala Leu
 115 120 125

Asp Leu Leu Ile Lys Leu Leu Gln Thr Phe Arg Ser Ser Arg Leu Met
 130 135 140

Asp Glu Phe Lys Ile Gly Glu Leu Phe Ser Lys Phe Tyr Gly Glu Leu
 145 150 155 160

Ala Leu Lys Lys Lys Ile Pro Asp Thr Val Leu Glu Lys Val Tyr Glu
 165 170 175

Leu Leu Gly Leu Leu Gly Glu Val His Pro Ser Glu Met Ile Asn Asn
 180 185 190

Ala Glu Asn Leu Phe Arg Ala Phe Leu Gly Glu Leu Lys Thr Gln Met
195 200 205

Thr Ser Ala Val Arg Glu Pro Lys Leu Pro Val Leu Ala Gly Cys Leu
210 215 220

Lys Gly Leu Ser Ser Leu Leu Cys Asn Phe Thr Lys Ser Met Glu Glu
225 230 235 240

Asp Pro Gln Thr Ser Arg Glu Ile Phe Asn Phe Val Leu Lys Ala Ile
245 250 255

Arg Pro Gln Ile Asp Leu Lys Arg Tyr Ala Val Pro Ser Ala Gly Leu
260 265 270

Arg Leu Phe Ala Leu His Ala Ser Gln Phe Ser Thr Cys Leu Leu Asp
275 280 285

Asn Tyr Val Ser Leu Phe Glu Val Leu Leu Lys Trp Cys Ala His Thr
290 295 300

Asn Val Glu Leu Lys Lys Ala Ala Leu Ser Ala Leu Glu Ser Phe Leu
305 310 315 320

Lys Gln Val Ser Asn Met Val Ala Lys Asn Ala Glu Met His Lys Asn
325 330 335

Lys Leu Gln Tyr Phe Met Glu Gln Phe Tyr Gly Ile Ile Arg Asn Val
340 345 350

Asp Ser Asn Asn Lys Glu Leu Ser Ile Ala Ile Arg Gly Tyr Gly Leu
355 360 365

Phe Ala Gly Pro Cys Lys Val Ile Asn Ala Lys Asp Val Asp Phe Met
370 375 380

Tyr Val Glu Leu Ile Gln Arg Cys Lys Gln Met Phe Leu Thr Gln Thr
385 390 395 400

Asp Thr Gly Asp Tyr Arg Val Tyr Gln Met Pro Ser Phe Leu Gln Ser
405 410 415

Val Ala Ser Val Leu Leu Tyr Leu Asp Thr Val Pro Glu Val Tyr Thr
420 425 430

Pro Val Leu Glu His Leu Val Val Met Gln Ile Asp Ser Phe Pro Gln
 435 440 445

Tyr Ser Pro Lys Met Gln Leu Val Cys Cys Arg Ala Ile Val Lys Val
 450 455 460

Phe Leu Ala Leu Ala Ala Lys Gly Pro Val Leu Arg Asn Cys Ile Ser
 465 470 475 480

Thr Val Val His Gln Gly Leu Ile Arg Ile Cys Ser Lys Pro Val Val
 485 490 495

Leu Pro Lys Gly Pro Glu Ser Glu Ser Glu Asp His Arg Ala Ser Gly
 500 505 510

Glu Val Arg Thr Gly Lys Trp Lys Val Pro Thr Tyr Lys Asp Tyr Val
 515 520 525

Asp Leu Phe Arg His Leu Leu Ser Ser Asp Gln Met Met Asp Ser Ile
 530 535 540

Leu Ala Asp Glu Ala Phe Phe Ser Val Asn Ser Ser Ser Glu Ser Leu
 545 550 555 560

Asn His Leu Leu Tyr Asp Glu Phe Val Lys Ser Val Leu Lys Ile Val
 565 570 575

Glu Lys Leu Asp Leu Thr Leu Glu Ile Gln Thr Val Gly Glu Gln Glu
 580 585 590

Asn Gly Asp Glu Ala Pro Gly Val Trp Met Ile Pro Thr Ser Asp Pro
 595 600 605

Ala Ala Asn Leu His Pro Ala Lys Pro Lys Asp Phe Ser Ala Phe Ile
 610 615 620

Asn Leu Val Glu Phe Cys Arg Glu Ile Leu Pro Glu Lys Gln Ala Glu
 625 630 635 640

Phe Phe Glu Pro Trp Val Tyr Ser Phe Ser Tyr Glu Leu Ile Leu Gln
 645 650 655

Ser Thr Arg Leu Pro Leu Ile Ser Gly Phe Tyr Lys Leu Leu Ser Ile
 660 665 670

Thr Val Arg Asn Ala Lys Lys Ile Lys Tyr Phe Glu Gly Val Ser Pro
 675 680 685

Lys Ser Leu Lys His Ser Pro Glu Asp Pro Glu Lys Tyr Ser Cys Phe
 690 695 700

Ala Leu Phe Val Lys Phe Gly Lys Glu Val Ala Val Lys Met Lys Gln
 705 710 715 720

Tyr Lys Asp Glu Leu Leu Ala Ser Cys Leu Thr Phe Leu Leu Ser Leu
 725 730 735

Pro His Asn Ile Ile Glu Leu Asp Val Arg Ala Tyr Val Pro Ala Leu
 740 745 750

Gln Met Ala Phe Lys Leu Gly Leu Ser Tyr Thr Pro Leu Ala Glu Val
 755 760 765

Gly Leu Asn Ala Leu Glu Glu Trp Ser Ile Tyr Ile Asp Arg His Val
 770 775 780

Met Gln Pro Tyr Tyr Lys Asp Ile Leu Pro Cys Leu Asp Gly Tyr Leu
 785 790 795 800

Lys Thr Ser Ala Leu Ser Asp Glu Thr Lys Asn Asn Trp Glu Val Ser
 805 810 815

Ala Leu Ser Arg Ala Ala Gln Lys Gly Phe Asn Lys Val Val Leu Lys
 820 825 830

His Leu Lys Lys Thr Lys Asn Leu Ser Ser Asn Glu Ala Ile Ser Leu
 835 840 845

Glu Glu Ile Arg Ile Arg Val Val Gln Met Leu Gly Ser Leu Gly Gly
 850 855 860

Gln Ile Asn Lys Asn Leu Leu Thr Val Thr Ser Ser Asp Glu Met Met
 865 870 875 880

Lys Ser Tyr Val Ala Trp Asp Arg Glu Lys Arg Leu Ser Phe Ala Val
 885 890 895

Pro Phe Arg Glu Met Lys Pro Val Ile Phe Leu Asp Val Phe Leu Pro
 900 905 910

Arg Val Thr Glu Leu Ala Leu Thr Ala Ser Asp Arg Gln Thr Lys Val

915

920

925

Ala Ala Cys Glu Leu Leu His Ser Met Val Met Phe Met Leu Gly Lys
 930 935 940

Ala Thr Gln Met Pro Glu Gly Gly Gln Gly Ala Pro Pro Met Tyr Gln
 945 950 955 960

Leu Tyr Lys Arg Thr Phe Pro Val Leu Leu Arg Leu Ala Cys Asp Val
 965 970 975

Asp Gln Val Thr Arg Gln Leu Tyr Glu Pro Leu Val Met Gln Leu Ile
 980 985 990

His Trp Phe Thr Asn Asn Lys Lys Phe Glu Ser Gln Asp Thr Val Ser
 995 1000 1005

Leu Leu Glu Ala Ile Leu Asp Gly Ile Val Asp Pro Val Asp Ser
 1010 1015 1020

Thr Leu Arg Asp Phe Cys Gly Arg Cys Ile Arg Glu Phe Leu Lys
 1025 1030 1035

Trp Ser Ile Lys Gln Ile Thr Pro Gln Gln Gln Glu Lys Ser Pro
 1040 1045 1050

Val Asn Thr Lys Ser Leu Phe Lys Arg Leu Tyr Ser Leu Ala Leu
 1055 1060 1065

His Pro Asn Ala Phe Lys Arg Leu Gly Ala Ser Leu Ala Phe Asn
 1070 1075 1080

Asn Ile Tyr Arg Glu Phe Arg Glu Glu Glu Ser Leu Val Glu Gln
 1085 1090 1095

Phe Val Phe Glu Ala Leu Val Ile Tyr Met Glu Ser Leu Ala Leu
 1100 1105 1110

Ala His Ala Asp Glu Lys Ser Leu Gly Thr Ile Gln Gln Cys Cys
 1115 1120 1125

Asp Ala Ile Asp His Leu Cys Arg Ile Ile Glu Lys Lys His Val
 1130 1135 1140

Ser Leu Asn Lys Ala Lys Lys Arg Arg Leu Pro Arg Gly Phe Pro
 1145 1150 1155

Pro Ser Ala Ser Leu Cys Leu Leu Asp Leu Val Lys Trp Leu Leu
1160 1165 1170

Ala His Cys Gly Arg Pro Gln Thr Glu Cys Arg His Lys Ser Ile
1175 1180 1185

Glu Leu Phe Tyr Lys Phe Val Pro Leu Leu Pro Gly Asn Arg Ser
1190 1195 1200

Pro Asn Leu Trp Leu Lys Asp Val Leu Lys Glu Glu Gly Val Ser
1205 1210 1215

Phe Leu Ile Asn Thr Phe Glu Gly Gly Gly Cys Gly Gln Pro Ser
1220 1225 1230

Gly Ile Leu Ala Gln Pro Thr Leu Leu Tyr Leu Arg Gly Pro Phe
1235 1240 1245

Ser Leu Gln Ala Thr Leu Cys Trp Leu Asp Leu Leu Leu Ala Ala
1250 1255 1260

Leu Glu Cys Tyr Asn Thr Phe Ile Gly Glu Arg Thr Val Gly Ala
1265 1270 1275

Leu Gln Val Leu Gly Thr Glu Ala Gln Ser Ser Leu Leu Lys Ala
1280 1285 1290

Val Ala Phe Phe Leu Glu Ser Ile Ala Met His Asp Ile Ile Ala
1295 1300 1305

Ala Glu Lys Cys Phe Gly Thr Gly Ala Ala Gly Asn Arg Thr Ser
1310 1315 1320

Pro Gln Glu Gly Glu Arg Tyr Asn Tyr Ser Lys Cys Thr Val Val
1325 1330 1335

Val Arg Ile Met Glu Phe Thr Thr Thr Leu Leu Asn Thr Ser Pro
1340 1345 1350

Glu Gly Trp Lys Leu Leu Lys Lys Asp Leu Cys Asn Thr His Leu
1355 1360 1365

Met Arg Val Leu Val Gln Thr Leu Cys Glu Pro Ala Ser Ile Gly
1370 1375 1380

Phe	Asn	Ile	Gly	Asp	Val	Gln	Val	Met	Ala	His	Leu	Pro	Asp	Val
1385						1390					1395			
Cys	Val	Asn	Leu	Met	Lys	Ala	Leu	Lys	Met	Ser	Pro	Tyr	Lys	Asp
1400						1405					1410			
Ile	Leu	Glu	Thr	His	Leu	Arg	Glu	Lys	Ile	Thr	Ala	Gln	Ser	Ile
1415						1420					1425			
Glu	Glu	Leu	Cys	Ala	Val	Asn	Leu	Tyr	Gly	Pro	Asp	Ala	Gln	Val
1430						1435					1440			
Asp	Arg	Ser	Arg	Leu	Ala	Ala	Val	Val	Ser	Ala	Cys	Lys	Gln	Leu
1445						1450					1455			
His	Arg	Ala	Gly	Leu	Leu	His	Asn	Ile	Leu	Pro	Ser	Gln	Ser	Thr
1460						1465					1470			
Asp	Leu	His	His	Ser	Val	Gly	Thr	Glu	Leu	Leu	Ser	Leu	Val	Tyr
1475						1480					1485			
Lys	Gly	Ile	Ala	Pro	Gly	Asp	Glu	Arg	Gln	Cys	Leu	Pro	Ser	Leu
1490						1495					1500			
Asp	Leu	Ser	Cys	Lys	Gln	Leu	Ala	Ser	Gly	Leu	Leu	Glu	Leu	Ala
1505						1510					1515			
Phe	Ala	Phe	Gly	Gly	Leu	Cys	Glu	Arg	Leu	Val	Ser	Leu	Leu	Leu
1520						1525					1530			
Asn	Pro	Ala	Val	Leu	Ser	Thr	Ala	Ser	Leu	Gly	Ser	Ser	Gln	Gly
1535						1540					1545			
Ser	Val	Ile	His	Phe	Ser	His	Gly	Glu	Tyr	Phe	Tyr	Ser	Leu	Phe
1550						1555					1560			
Ser	Glu	Thr	Ile	Asn	Thr	Glu	Leu	Leu	Lys	Asn	Leu	Asp	Leu	Ala
1565						1570					1575			
Val	Leu	Glu	Leu	Met	Gln	Ser	Ser	Val	Asp	Asn	Thr	Lys	Met	Val
1580						1585					1590			
Ser	Ala	Val	Leu	Asn	Gly	Met	Leu	Asp	Gln	Ser	Phe	Arg	Glu	Arg
1595						1600					1605			

Ala	Asn	Gln	Lys	His	Gln	Gly	Leu	Lys	Leu	Ala	Thr	Thr	Ile	Leu
1610						1615					1620			
Gln	His	Trp	Lys	Lys	Cys	Asp	Ser	Trp	Trp	Ala	Lys	Asp	Ser	Pro
1625						1630					1635			
Leu	Glu	Thr	Lys	Met	Ala	Val	Leu	Ala	Leu	Leu	Ala	Lys	Ile	Leu
1640						1645					1650			
Gln	Ile	Asp	Ser	Ser	Val	Ser	Phe	Asn	Thr	Ser	His	Gly	Ser	Phe
1655						1660					1665			
Pro	Glu	Val	Phe	Thr	Thr	Tyr	Ile	Ser	Leu	Leu	Ala	Asp	Thr	Lys
1670						1675					1680			
Leu	Asp	Leu	His	Leu	Lys	Gly	Gln	Ala	Val	Thr	Leu	Leu	Pro	Phe
1685						1690					1695			
Phe	Thr	Ser	Leu	Thr	Gly	Gly	Ser	Leu	Glu	Glu	Leu	Arg	Arg	Val
1700						1705					1710			
Leu	Glu	Gln	Leu	Ile	Val	Ala	His	Phe	Pro	Met	Gln	Ser	Arg	Glu
1715						1720					1725			
Phe	Pro	Pro	Gly	Thr	Pro	Arg	Phe	Asn	Asn	Tyr	Val	Asp	Cys	Met
1730						1735					1740			
Lys	Lys	Phe	Leu	Asp	Ala	Leu	Glu	Leu	Ser	Gln	Ser	Pro	Met	Leu
1745						1750					1755			
Leu	Glu	Leu	Met	Thr	Glu	Val	Leu	Cys	Arg	Glu	Gln	Gln	His	Val
1760						1765					1770			
Met	Glu	Glu	Leu	Phe	Gln	Ser	Ser	Phe	Arg	Arg	Ile	Ala	Arg	Arg
1775						1780					1785			
Gly	Ser	Cys	Val	Thr	Gln	Val	Gly	Leu	Leu	Glu	Ser	Val	Tyr	Glu
1790						1795					1800			
Met	Phe	Arg	Lys	Asp	Asp	Pro	Arg	Leu	Ser	Phe	Thr	Arg	Gln	Ser
1805						1810					1815			
Phe	Val	Asp	Arg	Ser	Leu	Leu	Thr	Leu	Leu	Trp	His	Cys	Ser	Leu
1820						1825					1830			
Asp	Ala	Leu	Arg	Glu	Phe	Phe	Ser	Thr	Ile	Val	Val	Asp	Ala	Ile

1835	1840	1845
Asp Val Leu Lys Ser Arg Phe Thr Lys Leu Asn Glu Ser Thr Phe 1850 1855 1860		
Asp Thr Gln Ile Thr Lys Lys Met Gly Tyr Tyr Lys Ile Leu Asp 1865 1870 1875		
Val Met Tyr Ser Arg Leu Pro Lys Asp Asp Val His Ala Lys Glu 1880 1885 1890		
Ser Lys Ile Asn Gln Val Phe His Gly Ser Cys Ile Thr Glu Gly 1895 1900 1905		
Asn Glu Leu Thr Lys Thr Leu Ile Lys Leu Cys Tyr Asp Ala Phe 1910 1915 1920		
Thr Glu Asn Met Ala Gly Glu Asn Gln Leu Leu Glu Arg Arg Arg 1925 1930 1935		
Leu Tyr His Cys Ala Ala Tyr Asn Cys Ala Ile Ser Val Ile Cys 1940 1945 1950		
Cys Val Phe Asn Glu Leu Lys Phe Tyr Gln Gly Phe Leu Phe Ser 1955 1960 1965		
Glu Lys Pro Glu Lys Asn Leu Leu Ile Phe Glu Asn Leu Ile Asp 1970 1975 1980		
Leu Lys Arg Arg Tyr Asn Phe Pro Val Glu Val Glu Val Pro Met 1985 1990 1995		
Glu Arg Lys Lys Lys Tyr Ile Glu Ile Arg Lys Glu Ala Arg Glu 2000 2005 2010		
Ala Ala Asn Gly Asp Ser Asp Gly Pro Ser Tyr Met Ser Ser Leu 2015 2020 2025		
Ser Tyr Leu Ala Asp Ser Thr Leu Ser Glu Glu Met Ser Gln Phe 2030 2035 2040		
Asp Phe Ser Thr Gly Val Gln Ser Tyr Ser Tyr Ser Ser Gln Asp 2045 2050 2055		
Pro Arg Pro Ala Thr Gly Arg Phe Arg Arg Arg Glu Gln Arg Asp 2060 2065 2070		

Pro Thr Val His Asp Asp Val Leu Glu Leu Glu Met Asp Glu Leu
 2075 2080 2085

Asn Arg His Glu Cys Met Ala Pro Leu Thr Ala Leu Val Lys His
 2090 2095 2100

Met His Arg Ser Leu Gly Pro Pro Gln Gly Glu Glu Asp Ser Val
 2105 2110 2115

Pro Arg Asp Leu Pro Ser Trp Met Lys Phe Leu His Gly Lys Leu
 2120 2125 2130

Gly Asn Pro Ile Val Pro Leu Asn Ile Arg Leu Phe Leu Ala Lys
 2135 2140 2145

Leu Val Ile Asn Thr Glu Glu Val Phe Arg Pro Tyr Ala Lys His
 2150 2155 2160

Trp Leu Ser Pro Leu Leu Gln Leu Ala Ala Ser Glu Asn Asn Gly
 2165 2170 2175

Gly Glu Gly Ile His Tyr Met Val Val Glu Ile Val Ala Thr Ile
 2180 2185 2190

Leu Ser Trp Thr Gly Leu Ala Thr Pro Thr Gly Val Pro Lys Asp
 2195 2200 2205

Glu Val Leu Ala Asn Arg Leu Leu Asn Phe Leu Met Lys His Val
 2210 2215 2220

Phe His Pro Lys Arg Ala Val Phe Arg His Asn Leu Glu Ile Ile
 2225 2230 2235

Lys Thr Leu Val Glu Cys Trp Lys Asp Cys Leu Ser Ile Pro Tyr
 2240 2245 2250

Arg Leu Ile Phe Glu Lys Phe Ser Gly Lys Asp Pro Asn Ser Lys
 2255 2260 2265

Asp Asn Ser Val Gly Ile Gln Leu Leu Gly Ile Val Met Ala Asn
 2270 2275 2280

Asp Leu Pro Pro Tyr Asp Pro Gln Cys Gly Ile Gln Ser Ser Glu
 2285 2290 2295

Tyr	Phe	Gln	Ala	Leu	Val	Asn	Asn	Met	Ser	Phe	Val	Arg	Tyr	Lys
2300						2305					2310			
Glu	Val	Tyr	Ala	Ala	Ala	Ala	Glu	Val	Leu	Gly	Leu	Ile	Leu	Arg
2315						2320					2325			
Tyr	Val	Met	Glu	Arg	Lys	Asn	Ile	Leu	Glu	Glu	Ser	Leu	Cys	Glu
2330						2335					2340			
Leu	Val	Ala	Lys	Gln	Leu	Lys	Gln	His	Gln	Asn	Thr	Met	Glu	Asp
2345						2350					2355			
Lys	Phe	Ile	Val	Cys	Leu	Asn	Lys	Val	Thr	Lys	Ser	Phe	Pro	Pro
2360						2365					2370			
Leu	Ala	Asp	Arg	Phe	Met	Asn	Ala	Val	Phe	Phe	Leu	Leu	Pro	Lys
2375						2380					2385			
Phe	His	Gly	Val	Leu	Lys	Thr	Leu	Cys	Leu	Glu	Val	Val	Leu	Cys
2390						2395					2400			
Arg	Val	Glu	Gly	Met	Thr	Glu	Leu	Tyr	Phe	Gln	Leu	Lys	Ser	Lys
2405						2410					2415			
Asp	Phe	Val	Gln	Val	Met	Arg	His	Arg	Asp	Asp	Glu	Arg	Gln	Lys
2420						2425					2430			
Val	Cys	Leu	Asp	Ile	Ile	Tyr	Lys	Met	Met	Pro	Lys	Leu	Lys	Pro
2435						2440					2445			
Val	Glu	Leu	Arg	Glu	Leu	Leu	Asn	Pro	Val	Val	Glu	Phe	Val	Ser
2450						2455					2460			
His	Pro	Ser	Thr	Thr	Cys	Arg	Glu	Gln	Met	Tyr	Asn	Ile	Leu	Met
2465						2470					2475			
Trp	Ile	His	Asp	Asn	Tyr	Arg	Asp	Pro	Glu	Ser	Glu	Thr	Asp	Asn
2480						2485					2490			
Asp	Ser	Gln	Glu	Ile	Phe	Lys	Leu	Ala	Lys	Asp	Val	Leu	Ile	Gln
2495						2500					2505			
Gly	Leu	Ile	Asp	Glu	Asn	Pro	Gly	Leu	Gln	Leu	Ile	Ile	Arg	Asn
2510						2515					2520			

Phe Trp Ser His Glu Thr Arg Leu Pro Ser Asn Thr Leu Asp Arg
 2525 2530 2535

 Leu Leu Ala Leu Asn Ser Leu Tyr Ser Pro Lys Ile Glu Val His
 2540 2545 2550

 Phe Leu Ser Leu Ala Thr Asn Phe Leu Leu Glu Met Thr Ser Met
 2555 2560 2565

 Ser Pro Asp Tyr Pro Asn Pro Met Phe Glu His Pro Leu Ser Glu
 2570 2575 2580

 Cys Glu Phe Gln Glu Tyr Thr Ile Asp Ser Asp Trp Arg Phe Arg
 2585 2590 2595

 Ser Thr Val Leu Thr Pro Met Phe Val Glu Thr Gln Ala Ser Gln
 2600 2605 2610

 Gly Thr Leu Gln Thr Arg Thr Gln Glu Gly Ser Leu Ser Ala Arg
 2615 2620 2625

 Trp Pro Val Ala Gly Gln Ile Arg Ala Thr Gln Gln Gln His Asp
 2630 2635 2640

 Phe Thr Leu Thr Gln Thr Ala Asp Gly Arg Ser Ser Phe Asp Trp
 2645 2650 2655

 Leu Thr Gly Ser Ser Thr Asp Pro Leu Val Asp His Thr Ser Pro
 2660 2665 2670

 Ser Ser Asp Ser Leu Leu Phe Ala His Lys Arg Ser Glu Arg Leu
 2675 2680 2685

 Gln Arg Ala Pro Leu Lys Ser Val Gly Pro Asp Phe Gly Lys Lys
 2690 2695 2700

 Arg Leu Gly Leu Pro Gly Asp Glu Val Asp Asn Lys Val Lys Gly
 2705 2710 2715

 Ala Ala Gly Arg Thr Asp Leu Leu Arg Leu Arg Arg Arg Phe Met
 2720 2725 2730

 Arg Asp Gln Glu Lys Leu Ser Leu Met Tyr Ala Arg Lys Gly Val
 2735 2740 2745

 Ala Glu Gln Lys Arg Glu Lys Glu Ile Lys Ser Glu Leu Lys Met

2750

2755

2760

Lys Gln Asp Ala Gln Val Val Leu Tyr Arg Ser Tyr Arg His Gly
 2765 2770 2775

Asp Leu Pro Asp Ile Gln Ile Lys His Ser Ser Leu Ile Thr Pro
 2780 2785 2790

Leu Gln Ala Val Ala Gln Arg Asp Pro Ile Ile Ala Lys Gln Leu
 2795 2800 2805

Phe Ser Ser Leu Phe Ser Gly Ile Leu Lys Glu Met Asp Lys Phe
 2810 2815 2820

Lys Thr Leu Ser Glu Lys Asn Asn Ile Thr Gln Lys Leu Leu Gln
 2825 2830 2835

Asp Phe Asn Arg Phe Leu Asn Thr Thr Phe Ser Phe Phe Pro Pro
 2840 2845 2850

Phe Val Ser Cys Ile Gln Asp Ile Ser Cys Gln His Ala Ala Leu
 2855 2860 2865

Leu Ser Leu Asp Pro Ala Ala Val Ser Ala Gly Cys Leu Ala Ser
 2870 2875 2880

Leu Gln Gln Pro Val Gly Ile Arg Leu Leu Glu Glu Ala Leu Leu
 2885 2890 2895

Arg Leu Leu Pro Ala Glu Leu Pro Ala Lys Arg Val Arg Gly Lys
 2900 2905 2910

Ala Arg Leu Pro Pro Asp Val Leu Arg Trp Val Glu Leu Ala Lys
 2915 2920 2925

Leu Tyr Arg Ser Ile Gly Glu Tyr Asp Val Leu Arg Gly Ile Phe
 2930 2935 2940

Thr Ser Glu Ile Gly Thr Lys Gln Ile Thr Gln Ser Ala Leu Leu
 2945 2950 2955

Ala Glu Ala Arg Ser Asp Tyr Ser Glu Ala Ala Lys Gln Tyr Asp
 2960 2965 2970

Glu Ala Leu Asn Lys Gln Asp Trp Val Asp Gly Glu Pro Thr Glu
 2975 2980 2985

Ala	Glu	Lys	Asp	Phe	Trp	Glu	Leu	Ala	Ser	Leu	Asp	Cys	Tyr	Asn
2990						2995					3000			
His	Leu	Ala	Glu	Trp	Lys	Ser	Leu	Glu	Tyr	Cys	Ser	Thr	Ala	Ser
3005						3010					3015			
Ile	Asp	Ser	Glu	Asn	Pro	Pro	Asp	Leu	Asn	Lys	Ile	Trp	Ser	Glu
3020						3025					3030			
Pro	Phe	Tyr	Gln	Glu	Thr	Tyr	Leu	Pro	Tyr	Met	Ile	Arg	Ser	Lys
3035						3040					3045			
Leu	Lys	Leu	Leu	Leu	Gln	Gly	Glu	Ala	Asp	Gln	Ser	Leu	Leu	Thr
3050						3055					3060			
Phe	Ile	Asp	Lys	Ala	Met	His	Gly	Glu	Leu	Gln	Lys	Ala	Ile	Leu
3065						3070					3075			
Glu	Leu	His	Tyr	Ser	Gln	Glu	Leu	Ser	Leu	Leu	Tyr	Leu	Leu	Gln
3080						3085					3090			
Asp	Asp	Val	Asp	Arg	Ala	Lys	Tyr	Tyr	Ile	Gln	Asn	Gly	Ile	Gln
3095						3100					3105			
Ser	Phe	Met	Gln	Asn	Tyr	Ser	Ser	Ile	Asp	Val	Leu	Leu	His	Gln
3110						3115					3120			
Ser	Arg	Leu	Thr	Lys	Leu	Gln	Ser	Val	Gln	Ala	Leu	Thr	Glu	Ile
3125						3130					3135			
Gln	Glu	Phe	Ile	Ser	Phe	Ile	Ser	Lys	Gln	Gly	Asn	Leu	Ser	Ser
3140						3145					3150			
Gln	Val	Pro	Leu	Lys	Arg	Leu	Leu	Asn	Thr	Trp	Thr	Asn	Arg	Tyr
3155						3160					3165			
Pro	Asp	Ala	Lys	Met	Asp	Pro	Met	Asn	Ile	Trp	Asp	Asp	Ile	Ile
3170						3175					3180			
Thr	Asn	Arg	Cys	Phe	Phe	Leu	Ser	Lys	Ile	Glu	Glu	Lys	Leu	Thr
3185						3190					3195			
Pro	Leu	Pro	Glu	Asp	Asn	Ser	Met	Asn	Val	Asp	Gln	Asp	Gly	Asp
3200						3205					3210			

Pro Ser Asp Arg Met Glu Val Gln Glu Gln Glu Glu Asp Ile Ser
 3215 3220 3225

Ser Leu Ile Arg Ser Cys Lys Phe Ser Met Lys Met Lys Met Ile
 3230 3235 3240

Asp Ser Ala Arg Lys Gln Asn Asn Phe Ser Leu Ala Met Lys Leu
 3245 3250 3255

Leu Lys Glu Leu His Lys Glu Ser Lys Thr Arg Asp Asp Trp Leu
 3260 3265 3270

Val Ser Trp Val Gln Ser Tyr Cys Arg Leu Ser His Cys Arg Ser
 3275 3280 3285

Arg Ser Gln Gly Cys Ser Glu Gln Val Leu Thr Val Leu Lys Thr
 3290 3295 3300

Val Ser Leu Leu Asp Glu Asn Asn Val Ser Ser Tyr Leu Ser Lys
 3305 3310 3315

Asn Ile Leu Ala Phe Arg Asp Gln Asn Ile Leu Leu Gly Thr Thr
 3320 3325 3330

Tyr Arg Ile Ile Ala Asn Ala Leu Ser Ser Glu Pro Ala Cys Leu
 3335 3340 3345

Ala Glu Ile Glu Glu Asp Lys Ala Arg Arg Ile Leu Glu Leu Ser
 3350 3355 3360

Gly Ser Ser Ser Glu Asp Ser Glu Lys Val Ile Ala Gly Leu Tyr
 3365 3370 3375

Gln Arg Ala Phe Gln His Leu Ser Glu Ala Val Gln Ala Ala Glu
 3380 3385 3390

Glu Glu Ala Gln Pro Pro Ser Trp Ser Cys Gly Pro Ala Ala Gly
 3395 3400 3405

Val Ile Asp Ala Tyr Met Thr Leu Ala Asp Phe Cys Asp Gln Gln
 3410 3415 3420

Leu Arg Lys Glu Glu Glu Asn Ala Ser Val Thr Asp Ser Ala Glu
 3425 3430 3435

Leu	Gln	Ala	Tyr	Pro	Ala	Leu	Val	Val	Glu	Lys	Met	Leu	Lys	Ala
3440						3445					3450			
Leu	Lys	Leu	Asn	Ser	Asn	Glu	Ala	Arg	Leu	Lys	Phe	Pro	Arg	Leu
3455						3460					3465			
Leu	Gln	Ile	Ile	Glu	Arg	Tyr	Pro	Glu	Glu	Thr	Leu	Ser	Leu	Met
3470						3475					3480			
Thr	Lys	Glu	Ile	Ser	Ser	Val	Pro	Cys	Trp	Gln	Phe	Ile	Ser	Trp
3485						3490					3495			
Ile	Ser	His	Met	Val	Ala	Leu	Leu	Asp	Lys	Asp	Gln	Ala	Val	Ala
3500						3505					3510			
Val	Gln	His	Ser	Val	Glu	Glu	Ile	Thr	Asp	Asn	Tyr	Pro	Gln	Ala
3515						3520					3525			
Ile	Val	Tyr	Pro	Phe	Ile	Ile	Ser	Ser	Glu	Ser	Tyr	Ser	Phe	Lys
3530						3535					3540			
Asp	Thr	Ser	Thr	Gly	His	Lys	Asn	Lys	Glu	Phe	Val	Ala	Arg	Ile
3545						3550					3555			
Lys	Ser	Lys	Leu	Asp	Gln	Gly	Gly	Val	Ile	Gln	Asp	Phe	Ile	Asn
3560						3565					3570			
Ala	Leu	Asp	Gln	Leu	Ser	Asn	Pro	Glu	Leu	Leu	Phe	Lys	Asp	Trp
3575						3580					3585			
Ser	Asn	Asp	Val	Arg	Ala	Glu	Leu	Ala	Lys	Thr	Pro	Val	Asn	Lys
3590						3595					3600			
Lys	Asn	Ile	Glu	Lys	Met	Tyr	Glu	Arg	Met	Tyr	Ala	Ala	Leu	Gly
3605						3610					3615			
Asp	Pro	Lys	Ala	Pro	Gly	Leu	Gly	Ala	Phe	Arg	Arg	Lys	Phe	Ile
3620						3625					3630			
Gln	Thr	Phe	Gly	Lys	Glu	Phe	Asp	Lys	His	Phe	Gly	Lys	Gly	Gly
3635						3640					3645			
Ser	Lys	Leu	Leu	Arg	Met	Lys	Leu	Ser	Asp	Phe	Asn	Asp	Ile	Thr
3650						3655					3660			
Asn	Met	Leu	Leu	Leu	Lys	Met	Asn	Lys	Asp	Ser	Lys	Pro	Pro	Gly

3665		3670		3675
Asn Leu Lys Glu Cys Ser Pro Trp Met Ser Asp Phe Lys Val Glu				
3680		3685		3690
Phe Leu Arg Asn Glu Leu Glu Ile Pro Gly Gln Tyr Asp Gly Arg				
3695		3700		3705
Gly Lys Pro Leu Pro Glu Tyr His Val Arg Ile Ala Gly Phe Asp				
3710		3715		3720
Glu Arg Val Thr Val Met Ala Ser Leu Arg Arg Pro Lys Arg Ile				
3725		3730		3735
Ile Ile Arg Gly His Asp Glu Arg Glu His Pro Phe Leu Val Lys				
3740		3745		3750
Gly Gly Glu Asp Leu Arg Gln Asp Gln Arg Val Glu Gln Leu Phe				
3755		3760		3765
Gln Val Met Asn Gly Ile Leu Ala Gln Asp Ser Ala Cys Ser Gln				
3770		3775		3780
Arg Ala Leu Gln Leu Arg Thr Tyr Ser Val Val Pro Met Thr Ser				
3785		3790		3795
Arg Leu Gly Leu Ile Glu Trp Leu Glu Asn Thr Val Thr Leu Lys				
3800		3805		3810
Asp Leu Leu Leu Asn Thr Met Ser Gln Glu Glu Lys Ala Ala Tyr				
3815		3820		3825
Leu Ser Asp Pro Arg Ala Pro Pro Cys Glu Tyr Lys Asp Trp Leu				
3830		3835		3840
Thr Lys Met Ser Gly Lys His Asp Val Gly Ala Tyr Met Leu Met				
3845		3850		3855
Tyr Lys Gly Ala Asn Arg Thr Glu Thr Val Thr Ser Phe Arg Lys				
3860		3865		3870
Arg Glu Ser Lys Val Pro Ala Asp Leu Leu Lys Arg Ala Phe Val				
3875		3880		3885
Arg Met Ser Thr Ser Pro Glu Ala Phe Leu Ala Leu Arg Ser His				
3890		3895		3900

Phe	Ala	Ser	Ser	His	Ala	Leu	Ile	Cys	Ile	Ser	His	Trp	Ile	Leu
3905						3910					3915			
Gly	Ile	Gly	Asp	Arg	His	Leu	Asn	Asn	Phe	Met	Val	Ala	Met	Glu
3920						3925					3930			
Thr	Gly	Gly	Val	Ile	Gly	Ile	Asp	Phe	Gly	His	Ala	Phe	Gly	Ser
3935						3940					3945			
Ala	Thr	Gln	Phe	Leu	Pro	Val	Pro	Glu	Leu	Met	Pro	Phe	Arg	Leu
3950						3955					3960			
Thr	Arg	Gln	Phe	Ile	Asn	Leu	Met	Leu	Pro	Met	Lys	Glu	Thr	Gly
3965						3970					3975			
Leu	Met	Tyr	Ser	Ile	Met	Val	His	Ala	Leu	Arg	Ala	Phe	Arg	Ser
3980						3985					3990			
Asp	Pro	Gly	Leu	Leu	Thr	Asn	Thr	Met	Asp	Val	Phe	Val	Lys	Glu
3995						4000					4005			
Pro	Ser	Phe	Asp	Trp	Lys	Asn	Phe	Glu	Gln	Lys	Met	Leu	Lys	Lys
4010						4015					4020			
Gly	Gly	Ser	Trp	Ile	Gln	Glu	Ile	Asn	Val	Ala	Glu	Lys	Asn	Trp
4025						4030					4035			
Tyr	Pro	Arg	Gln	Lys	Ile	Cys	Tyr	Ala	Lys	Arg	Lys	Leu	Ala	Gly
4040						4045					4050			
Ala	Asn	Pro	Ala	Val	Ile	Thr	Cys	Asp	Glu	Leu	Leu	Leu	Gly	His
4055						4060					4065			
Glu	Lys	Ala	Pro	Ala	Phe	Arg	Asp	Tyr	Val	Ala	Val	Ala	Arg	Gly
4070						4075					4080			
Ser	Lys	Asp	His	Asn	Ile	Arg	Ala	Gln	Glu	Pro	Glu	Ser	Gly	Leu
4085						4090					4095			
Ser	Glu	Glu	Thr	Gln	Val	Lys	Cys	Leu	Met	Asp	Gln	Ala	Thr	Asp
4100						4105					4110			
Pro	Asn	Ile	Leu	Gly	Arg	Thr	Trp	Glu	Gly	Trp	Glu	Pro	Trp	Met
4115						4120					4125			

<210> 4

<211> 21

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(21)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (11)..(11)

<223> PHOSPHORYLATION at T2609

<400> 4

Ser	Thr	Val	Leu	Thr	Pro	Met	Phe	Val	Glu	Thr	Gln	Ala	Ser	Gln	Gly
1				5					10					15	

Thr	Leu	Gln	Thr	Arg
			20	

<210> 5

<211> 29

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(29)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (13)..(13)

<223> PHOSPHORYLATION at S2056

<400> 5

Asp	Phe	Ser	Thr	Gly	Val	Gln	Ser	Tyr	Ser	Tyr	Ser	Ser	Gln	Asp	Pro
1				5				10					15		

Arg	Pro	Ala	Thr	Gly	Arg	Phe	Arg	Arg	Arg	Glu	Gln	Arg
			20				25					

<210> 6

<211> 303

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(303)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (177)..(177)

<223> PHOSPHORYLATION at S2056

<400> 6

Met Tyr Ser Arg Leu Pro Lys Asp Asp Val His Ala Lys Glu Ser Lys
 1 5 10 15

Ile Asn Gln Val Phe His Gly Ser Cys Ile Thr Glu Gly Asn Glu Leu
 20 25 30

Thr Lys Thr Leu Ile Lys Leu Cys Tyr Asp Ala Phe Thr Glu Asn Met
 35 40 45

Ala Gly Glu Asn Gln Leu Leu Glu Arg Arg Arg Leu Tyr His Cys Ala
 50 55 60

Ala Tyr Asn Cys Ala Ile Ser Val Ile Cys Cys Val Phe Asn Glu Leu
 65 70 75 80

Lys Phe Tyr Gln Gly Phe Leu Phe Ser Glu Lys Pro Glu Lys Asn Leu
 85 90 95

Leu Ile Phe Glu Asn Leu Ile Asp Leu Lys Arg Arg Tyr Asn Phe Pro
 100 105 110

Val Glu Val Glu Val Pro Met Glu Arg Lys Lys Lys Tyr Ile Glu Ile
 115 120 125

Arg Lys Glu Ala Arg Glu Ala Ala Asn Gly Asp Ser Asp Gly Pro Ser
 130 135 140

Tyr Met Ser Ser Leu Ser Tyr Leu Ala Asp Ser Thr Leu Ser Glu Glu
 145 150 155 160

Met Ser Gln Phe Asp Phe Ser Thr Gly Val Gln Ser Tyr Ser Tyr Ser
 165 170 175

Ser Gln Asp Pro Arg Pro Ala Thr Gly Arg Phe Arg Arg Arg Glu Gln
 180 185 190

Arg Asp Pro Thr Val His Asp Asp Val Leu Glu Leu Glu Met Asp Glu
 195 200 205

Leu Asn Arg His Glu Cys Met Ala Pro Leu Thr Ala Leu Val Lys His
210 215 220

Met His Arg Ser Leu Gly Pro Pro Gln Gly Glu Glu Asp Ser Val Pro
225 230 235 240

Arg Asp Leu Pro Ser Trp Met Lys Phe Leu His Gly Lys Leu Gly Asn
245 250 255

Pro Ile Val Pro Leu Asn Ile Arg Leu Phe Leu Ala Lys Leu Val Ile
260 265 270

Asn Thr Glu Glu Val Phe Arg Pro Tyr Ala Lys His Trp Leu Ser Pro
275 280 285

Leu Leu Gln Leu Ala Ala Ser Glu Asn Asn Gly Gly Glu Gly Ile
290 295 300

<210> 7

<211> 388

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(388)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (177)..(177)

<223> PHOSPHORYLATION at S2056

<400> 7

Met Tyr Ser Arg Leu Pro Lys Asp Asp Val His Ala Lys Glu Ser Lys
1 5 10 15

Ile Asn Gln Val Phe His Gly Ser Cys Ile Thr Glu Gly Asn Glu Leu
20 25 30

Thr Lys Thr Leu Ile Lys Leu Cys Tyr Asp Ala Phe Thr Glu Asn Met
35 40 45

Ala Gly Glu Asn Gln Leu Leu Glu Arg Arg Arg Leu Tyr His Cys Ala
50 55 60

Ala Tyr Asn Cys Ala Ile Ser Val Ile Cys Cys Val Phe Asn Glu Leu
65 70 75 80

23

Ala Thr Pro Thr Gly Val Pro Lys Asp Glu Val Leu Ala Asn Arg Leu
 325 330 335

Leu Asn Phe Leu Met Lys His Val Phe His Pro Lys Arg Ala Val Phe
 340 345 350

Arg His Asn Leu Glu Ile Ile Lys Thr Leu Val Glu Cys Trp Lys Asp
 355 360 365

Cys Leu Ser Ile Pro Tyr Arg Leu Ile Phe Glu Lys Phe Ser Gly Lys
 370 375 380

Asp Pro Asn Ser
 385

<210> 8

<211> 821

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(821)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (177)..(177)

<223> PHOSPHORYLATION at S2056

<220>

<221> MOD_RES

<222> (730)..(730)

<223> PHOSPHORYLATION at T2609

<400> 8

Met Tyr Ser Arg Leu Pro Lys Asp Asp Val His Ala Lys Glu Ser Lys
 1 5 10 15

Ile Asn Gln Val Phe His Gly Ser Cys Ile Thr Glu Gly Asn Glu Leu
 20 25 30

Thr Lys Thr Leu Ile Lys Leu Cys Tyr Asp Ala Phe Thr Glu Asn Met
 35 40 45

Ala Gly Glu Asn Gln Leu Leu Glu Arg Arg Arg Leu Tyr His Cys Ala
 50 55 60

Ala Tyr Asn Cys Ala Ile Ser Val Ile Cys Cys Val Phe Asn Glu Leu

65					70						75				80
Lys	Phe	Tyr	Gln	Gly	Phe	Leu	Phe	Ser	Glu	Lys	Pro	Glu	Lys	Asn	Leu
				85					90					95	
Leu	Ile	Phe	Glu	Asn	Leu	Ile	Asp	Leu	Lys	Arg	Arg	Tyr	Asn	Phe	Pro
			100					105					110		
Val	Glu	Val	Glu	Val	Pro	Met	Glu	Arg	Lys	Lys	Lys	Tyr	Ile	Glu	Ile
		115					120					125			
Arg	Lys	Glu	Ala	Arg	Glu	Ala	Ala	Asn	Gly	Asp	Ser	Asp	Gly	Pro	Ser
		130				135					140				
Tyr	Met	Ser	Ser	Leu	Ser	Tyr	Leu	Ala	Asp	Ser	Thr	Leu	Ser	Glu	Glu
145					150					155				160	
Met	Ser	Gln	Phe	Asp	Phe	Ser	Thr	Gly	Val	Gln	Ser	Tyr	Ser	Tyr	Ser
				165					170					175	
Ser	Gln	Asp	Pro	Arg	Pro	Ala	Thr	Gly	Arg	Phe	Arg	Arg	Arg	Glu	Gln
			180					185					190		
Arg	Asp	Pro	Thr	Val	His	Asp	Asp	Val	Leu	Glu	Leu	Glu	Met	Asp	Glu
		195					200					205			
Leu	Asn	Arg	His	Glu	Cys	Met	Ala	Pro	Leu	Thr	Ala	Leu	Val	Lys	His
	210					215					220				
Met	His	Arg	Ser	Leu	Gly	Pro	Pro	Gln	Gly	Glu	Glu	Asp	Ser	Val	Pro
225					230					235				240	
Arg	Asp	Leu	Pro	Ser	Trp	Met	Lys	Phe	Leu	His	Gly	Lys	Leu	Gly	Asn
				245					250					255	
Pro	Ile	Val	Pro	Leu	Asn	Ile	Arg	Leu	Phe	Leu	Ala	Lys	Leu	Val	Ile
			260					265					270		
Asn	Thr	Glu	Glu	Val	Phe	Arg	Pro	Tyr	Ala	Lys	His	Trp	Leu	Ser	Pro
		275					280					285			
Leu	Leu	Gln	Leu	Ala	Ala	Ser	Glu	Asn	Asn	Gly	Gly	Glu	Gly	Ile	His
	290					295					300				
Tyr	Met	Val	Val	Glu	Ile	Val	Ala	Thr	Ile	Leu	Ser	Trp	Thr	Gly	Leu
305					310					315				320	

Ala Thr Pro Thr Gly Val Pro Lys Asp Glu Val Leu Ala Asn Arg Leu
 325 330 335

Leu Asn Phe Leu Met Lys His Val Phe His Pro Lys Arg Ala Val Phe
 340 345 350

Arg His Asn Leu Glu Ile Ile Lys Thr Leu Val Glu Cys Trp Lys Asp
 355 360 365

Cys Leu Ser Ile Pro Tyr Arg Leu Ile Phe Glu Lys Phe Ser Gly Lys
 370 375 380

Asp Pro Asn Ser Lys Asp Asn Ser Val Gly Ile Gln Leu Leu Gly Ile
 385 390 395 400

Val Met Ala Asn Asp Leu Pro Pro Tyr Asp Pro Gln Cys Gly Ile Gln
 405 410 415

Ser Ser Glu Tyr Phe Gln Ala Leu Val Asn Asn Met Ser Phe Val Arg
 420 425 430

Tyr Lys Glu Val Tyr Ala Ala Ala Glu Val Leu Gly Leu Ile Leu
 435 440 445

Arg Tyr Val Met Glu Arg Lys Asn Ile Leu Glu Glu Ser Leu Cys Glu
 450 455 460

Leu Val Ala Lys Gln Leu Lys Gln His Gln Asn Thr Met Glu Asp Lys
 465 470 475 480

Phe Ile Val Cys Leu Asn Lys Val Thr Lys Ser Phe Pro Pro Leu Ala
 485 490 495

Asp Arg Phe Met Asn Ala Val Phe Phe Leu Leu Pro Lys Phe His Gly
 500 505 510

Val Leu Lys Thr Leu Cys Leu Glu Val Val Leu Cys Arg Val Glu Gly
 515 520 525

Met Thr Glu Leu Tyr Phe Gln Leu Lys Ser Lys Asp Phe Val Gln Val
 530 535 540

Met Arg His Arg Asp Asp Glu Arg Gln Lys Val Cys Leu Asp Ile Ile
 545 550 555 560

Thr	Asp	Pro	Leu	Val	Asp	His	Thr	Ser	Pro	Ser	Ser	Asp	Ser	Leu	Leu
785					790					795					800

Phe Ala His Lys Arg Ser Glu Arg Leu Gln Arg Ala Pro Leu Lys Ser
805 810 815

Val Gly Pro Asp Phe
820

<210> 9

<211> 440

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1) . . (440)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (349) .. (349)

<223> PHOSPHORYLATION at T2609

<400> 9

Ser Gly Lys Asp Pro Asn Ser Lys Asp Asn Ser Val Gly Ile Gln Leu
1 5 10 15

Leu Gly Ile Val Met Ala Asn Asp Leu Pro Pro Tyr Asp Pro Gln Cys
20 25 30

Gly Ile Gln Ser Ser Glu Tyr Phe Gln Ala Leu Val Asn Asn Met Ser
35 40 45

Phe Val Arg Tyr Lys Glu Val Tyr Ala Ala Ala Ala Glu Val Leu Gly
50 55 60

Leu Ile Leu Arg Tyr Val Met Glu Arg Lys Asn Ile Leu Glu Glu Ser
65 70 75 80

Leu Cys Glu Leu Val Ala Lys Gln Leu Lys Gln His Gln Asn Thr Met
85 90 95

Glu Asp Lys Phe Ile Val Cys Leu Asn Lys Val Thr Lys Ser Phe Pro
100 105 110

Pro Leu Ala Asp Arg Phe Met Asn Ala Val Phe Phe Leu Leu Pro Lys
115 120 125

Phe His Gly Val Leu Lys Thr Leu Cys Leu Glu Val Val Leu Cys Arg
130 135 140

Val Glu Gly Met Thr Glu Leu Tyr Phe Gln Leu Lys Ser Lys Asp Phe
 145 150 155 160

Val Gln Val Met Arg His Arg Asp Asp Glu Arg Gln Lys Val Cys Leu
 165 170 175

Asp Ile Ile Tyr Lys Met Met Pro Lys Leu Lys Pro Val Glu Leu Arg
 180 185 190

Glu Leu Leu Asn Pro Val Val Glu Phe Val Ser His Pro Ser Thr Thr
 195 200 205

Cys Arg Glu Gln Met Tyr Asn Ile Leu Met Trp Ile His Asp Asn Tyr
 210 215 220

Arg Asp Pro Glu Ser Glu Thr Asp Asn Asp Ser Gln Glu Ile Phe Lys
 225 230 235 240

Leu Ala Lys Asp Val Leu Ile Gln Gly Leu Ile Asp Glu Asn Pro Gly
 245 250 255

Leu Gln Leu Ile Ile Arg Asn Phe Trp Ser His Glu Thr Arg Leu Pro
 260 265 270

Ser Asn Thr Leu Asp Arg Leu Leu Ala Leu Asn Ser Leu Tyr Ser Pro
 275 280 285

Lys Ile Glu Val His Phe Leu Ser Leu Ala Thr Asn Phe Leu Leu Glu
 290 295 300

Met Thr Ser Met Ser Pro Asp Tyr Pro Asn Pro Met Phe Glu His Pro
 305 310 315 320

Leu Ser Glu Cys Glu Phe Gln Glu Tyr Thr Ile Asp Ser Asp Trp Arg
 325 330 335

Phe Arg Ser Thr Val Leu Thr Pro Met Phe Val Glu Thr Gln Ala Ser
 340 345 350

Gln Gly Thr Leu Gln Thr Arg Thr Gln Glu Gly Ser Leu Ser Ala Arg
 355 360 365

Trp Pro Val Ala Gly Gln Ile Arg Ala Thr Gln Gln Gln His Asp Phe
 370 375 380

Thr Leu Thr Gln Thr Ala Asp Gly Arg Ser Ser Phe Asp Trp Leu Thr
 385 390 395 400

Gly Ser Ser Thr Asp Pro Leu Val Asp His Thr Ser Pro Ser Ser Asp
 405 410 415

Ser Leu Leu Phe Ala His Lys Arg Ser Glu Arg Leu Gln Arg Ala Pro
 420 425 430

Leu Lys Ser Val Gly Pro Asp Phe
 435 440

<210> 10
 <211> 200
 <212> PRT
 <213> Artificial Sequence

<220>
 <221> PEPTIDE
 <222> (1)..(200)
 <223> HUMAN GENETIC ORIGIN

<220>
 <221> MOD_RES
 <222> (109)..(109)
 <223> PHOSPHORYLATION at T2609

<400> 10

Leu Ala Lys Asp Val Leu Ile Gln Gly Leu Ile Asp Glu Asn Pro Gly
 1 5 10 15

Leu Gln Leu Ile Ile Arg Asn Phe Trp Ser His Glu Thr Arg Leu Pro
 20 25 30

Ser Asn Thr Leu Asp Arg Leu Leu Ala Leu Asn Ser Leu Tyr Ser Pro
 35 40 45

Lys Ile Glu Val His Phe Leu Ser Leu Ala Thr Asn Phe Leu Leu Glu
 50 55 60

Met Thr Ser Met Ser Pro Asp Tyr Pro Asn Pro Met Phe Glu His Pro
 65 70 75 80

Leu Ser Glu Cys Glu Phe Gln Glu Tyr Thr Ile Asp Ser Asp Trp Arg
 85 90 95

Phe Arg Ser Thr Val Leu Thr Pro Met Phe Val Glu Thr Gln Ala Ser
 100 105 110

Gln Gly Thr Leu Gln Thr Arg Thr Gln Glu Gly Ser Leu Ser Ala Arg
 115 120 125

Trp Pro Val Ala Gly Gln Ile Arg Ala Thr Gln Gln Gln His Asp Phe
 130 135 140

Thr Leu Thr Gln Thr Ala Asp Gly Arg Ser Ser Phe Asp Trp Leu Thr
 145 150 155 160

Gly Ser Ser Thr Asp Pro Leu Val Asp His Thr Ser Pro Ser Ser Asp
 165 170 175

Ser Leu Leu Phe Ala His Lys Arg Ser Glu Arg Leu Gln Arg Ala Pro
 180 185 190

Leu Lys Ser Val Gly Pro Asp Phe
 195 200

<210> 11

<211> 428

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(428)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (335)..(335)

<223> PHOSPHORYLATION at T2609

<400> 11

Gln Leu Leu Gly Ile Val Met Ala Asn Asp Leu Pro Pro Tyr Asp Pro
 1 5 10 15

Gln Cys Gly Ile Gln Ser Ser Glu Tyr Phe Gln Ala Leu Val Asn Asn
 20 25 30

Met Ser Phe Val Arg Tyr Lys Glu Val Tyr Ala Ala Ala Ala Glu Val
 35 40 45

Leu Gly Leu Ile Leu Arg Tyr Val Met Glu Arg Lys Asn Ile Leu Glu
 50 55 60

Glu Ser Leu Cys Glu Leu Val Ala Lys Gln Leu Lys Gln His Gln Asn
 65 70 75 80

Thr Met Glu Asp Lys Phe Ile Val Cys Leu Asn Lys Val Thr Lys Ser
85 90 95

Phe Pro Pro Leu Ala Asp Arg Phe Met Asn Ala Val Phe Phe Leu Leu
100 105 110

Pro Lys Phe His Gly Val Leu Lys Thr Leu Cys Leu Glu Val Val Leu
115 120 125

Cys Arg Val Glu Gly Met Thr Glu Leu Tyr Phe Gln Leu Lys Ser Lys
130 135 140

Asp Phe Val Gln Val Met Arg His Arg Asp Asp Glu Arg Gln Lys Val
145 150 155 160

Cys Leu Asp Ile Ile Tyr Lys Met Met Pro Lys Leu Lys Pro Val Glu
165 170 175

Leu Arg Glu Leu Leu Asn Pro Val Val Glu Phe Val Ser His Pro Ser
180 185 190

Thr Thr Cys Arg Glu Gln Met Tyr Asn Ile Leu Met Trp Ile His Asp
195 200 205

Asn Tyr Arg Asp Pro Glu Ser Glu Thr Asp Asn Asp Ser Gln Glu Ile
210 215 220

Phe Lys Leu Ala Lys Asp Val Leu Ile Gln Gly Leu Ile Asp Glu Asn
225 230 235 240

Pro Gly Leu Gln Leu Ile Ile Arg Asn Phe Trp Ser His Glu Thr Arg
245 250 255

Leu Pro Ser Asn Thr Leu Asp Arg Leu Leu Ala Leu Asn Ser Leu Tyr
260 265 270

Ser Pro Lys Ile Glu Val His Phe Leu Ser Leu Ala Thr Asn Phe Leu
275 280 285

Leu Glu Met Thr Ser Met Ser Pro Asp Tyr Pro Asn Pro Met Phe Glu
290 295 300

His Pro Leu Ser Glu Cys Glu Phe Gln Glu Tyr Thr Ile Asp Ser Asp
305 310 315 320

Trp Arg Phe Arg Ser Thr Val Leu Thr Pro Met Phe Val Glu Thr Gln
325 330 335

Ala Ser Gln Gly Thr Leu Gln Thr Arg Thr Gln Glu Gly Ser Leu Ser
340 345 350

Ala Arg Trp Pro Val Ala Gly Gln Ile Arg Ala Thr Gln Gln Gln His
355 360 365

Asp Phe Thr Leu Thr Gln Thr Ala Asp Gly Arg Ser Ser Phe Asp Trp
370 375 380

Leu Thr Gly Ser Ser Thr Asp Pro Leu Val Asp His Thr Ser Pro Ser
385 390 395 400

Ser Asp Ser Leu Leu Phe Ala His Lys Arg Ser Glu Arg Leu Gln Arg
405 410 415

Ala Pro Leu Lys Ser Val Gly Pro Asp Phe Gly Lys
420 425

<210> 12

<211> 273

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1) .. (273)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (180) .. (180)

<223> PHOSPHORYLATION at T2609

<400> 12

Glu Arg Gln Lys Val Cys Leu Asp Ile Ile Tyr Lys Met Met Pro Lys
1 5 10 15

Leu Lys Pro Val Glu Leu Arg Glu Leu Leu Asn Pro Val Val Glu Phe
20 25 30

Val Ser His Pro Ser Thr Thr Cys Arg Glu Gln Met Tyr Asn Ile Leu
35 40 45

Met Trp Ile His Asp Asn Tyr Arg Asp Pro Glu Ser Glu Thr Asp Asn
50 55 60

Asp Ser Gln Glu Ile Phe Lys Leu Ala Lys Asp Val Leu Ile Gln Gly
65 70 75 80

Leu Ile Asp Glu Asn Pro Gly Leu Gln Leu Ile Ile Arg Asn Phe Trp
85 90 95

Ser His Glu Thr Arg Leu Pro Ser Asn Thr Leu Asp Arg Leu Leu Ala
100 105 110

Leu Asn Ser Leu Tyr Ser Pro Lys Ile Glu Val His Phe Leu Ser Leu
115 120 125

Ala Thr Asn Phe Leu Leu Glu Met Thr Ser Met Ser Pro Asp Tyr Pro
130 135 140

Asn Pro Met Phe Glu His Pro Leu Ser Glu Cys Glu Phe Gln Glu Tyr
145 150 155 160

Thr Ile Asp Ser Asp Trp Arg Phe Arg Ser Thr Val Leu Thr Pro Met
165 170 175

Phe Val Glu Thr Gln Ala Ser Gln Gly Thr Leu Gln Thr Arg Thr Gln
180 185 190

Glu Gly Ser Leu Ser Ala Arg Trp Pro Val Ala Gly Gln Ile Arg Ala
195 200 205

Thr Gln Gln Gln His Asp Phe Thr Leu Thr Gln Thr Ala Asp Gly Arg
210 215 220

Ser Ser Phe Asp Trp Leu Thr Gly Ser Ser Thr Asp Pro Leu Val Asp
225 230 235 240

His Thr Ser Pro Ser Ser Asp Ser Leu Leu Phe Ala His Lys Arg Ser
245 250 255

Glu Arg Leu Gln Arg Ala Pro Leu Lys Ser Val Gly Pro Asp Phe Gly
260 265 270

Lys

<210> 13

<211> 140

<212> PRT

<213> Artificial Sequence

<220>

<221> PEPTIDE

<222> (1)..(140)

<223> HUMAN GENETIC ORIGIN

<220>

<221> MOD_RES

<222> (49)..(49)

<223> PHOSPHORYLATION at T2609

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Thr Gln Ala Ser Gln Gly Thr Leu Gln Thr Arg Thr Gln Glu Gly Ser
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Asp Trp Leu Thr Gly Ser Ser Thr Asp Pro Leu Val Asp His Thr Ser
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gaaggatggg agccctggat gtgaggtctg tgggagctctg cagatagaaa gcattacatt 12480
gtttaaagaa tctactatac tttggttggc agcattccat gagctgattt tcctgaaaca 12540
ctaaagagaa atgtcttttg tgctacagtt tcgtagcatg agtttaaatc aagattatga 12600
tgagtaaatg tgtatgggtt aaatcaaaga taagggtata gtaacatcaa agattaggtg 12660
aggtttatag aaagatagat atccaggctt accaaagtat taagtcaaga atataatatg 12720
tgatcagctt tcaaagcatt tacaagtgt gcaagttagt gaaacagctg tctccgtaaa 12780
tgaggagaaat gtggggaagc cttggaatgc ccttctgggt ctggcacatt ggaaagcaca 12840
ctcagaaggc ttcataacca agattttggg agagtaaagc taagtatagt tgatgtaaca 12900
ttgtagaagc agcataggaa caataagaac aataggtaaa gctataatta tggcttatat 12960
ttagaaatga ctgcatttga tatttttagga tatttttcta ggttttttcc tttcatttta 13020
ttctcttcta gttttgacat tttatgatag atttgctctc tagaaggaaa cgtctttatt 13080

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taggagggca aaaatttttg tcatagcatt cacttttgct attccaatct acaactggaa 13140
gatacataaa agtgctttgc attgaatttg ggataacttc aaaaatccca tggttgttgt 13200
tagggatagt actaagcatt tcagttccag gagaataaaa gaaattccta tttgaaatga 13260
attcctcatt tggaggaaaa aaagcatgca ttctagcaca acaagatgaa attatggaat 13320
acaaaagtgg ctcttccca tgtgcagtc ctgtccccc cgcagtc tccacacca 13380
aactgtttct gattggcttt tagctttttg ttgttttttt ttttccttct aacacttgta 13440
tttgagggct cttctgtgat ttgagaagt atactcttga gtgtttaata aagttttttt 13500
ccaaaagta 13509

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<210> 16

<211> 63

<212> DNA

<213> Artificial Sequence

<220>

<223> cDNA sequence encoding the 2599-2619 peptide

<220>

<221> CDS

<222> (1)..(63)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (31)..(33)

<223> encodes T2609 residue

<400> 16

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agtactgttc tcactccgat gtttgtggag acccaggcct cccagggcac tctccagacc 60
cgt 63

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<210> 17

<211> 87

<212> DNA

<213> Artificial sequence

<220>

<223> cDNA sequence encoding the 2044-2072 peptide

<220>

<221> CDS

<222> (1)..(87)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (37)..(37)

<223> encodes S2056 residue

<400> 17

gattttctcaa ccggagttca gagctattca tacagctccc aagaccctag acctgccact 60

ggtcggttttc ggagacggga gcagcgg 87

<210> 18

<211> 909

<212> DNA

<213> Artificial Sequence

<220>

<223> cDNA sequence encoding the 1879-2182 peptide

<220>

<221> CDS

<222> (1)..(909)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (529)..(529)

<223> encodes S2056 residue

<400> 18

atgtattctc gccttcccaa agatgatgtt catgctaagg aatcaaaaat taatcaagtt 60

ttccatggct cgtgtattac agaaggaaat gaacttacia agacattgat taaattgtgc 120

tacgatgcat ttacagagaa catggcagga gagaatcagc tgctggagag gagaagactt 180

taccattgtg cagcatacaa ctgcgccata tctgtcatct gctgtgtctt caatgagtta 240

aaattttacc aagggttttct gtttagtgaa aaaccagaaa agaacttgct tatttttgaa 300

aatctgatcg acctgaagcg ccgctataat tttcctgtag aagttgaggt tcctatggaa 360

agaaagaaaa agtacattga aattaggaaa gaagccagag aagcagcaaa tggggattca 420

gatggctcctt cctatatgtc ttccctgtca tatttggcag acagtaccct gaggtaggaa 480

atgagtcaat ttgatttctc aaccggagtt cagagctatt catacagctc ccaagaccct 540

agacctgcca ctggtcgttt tcggagacgg gagcagcggg accccacggg gcatgatgat 600

gtgctggagc tggagatgga cgagctcaat cggcatgagt gcatggcgcc cctgacggcc 660

ctggtcaagc acatgcacag aagcctgggc ccgcctcaag gagaagagga ttcagtgcca 720

agagatcttc cttcttgat gaaattcctc catggcaaac tgggaaatcc aatagtacca 780

ttaaatatcc gtctcttctt agccaagctt gttattaata cagaagaggt ctttcgccct 840

tacgcgaagc actggcttag ccccttgctg cagctggctg cttctgaaaa caatggagga 900

gaaggaatt 909

<210> 19

<211> 1164

<212> DNA

<213> Artificial sequence

<220>

<223> Cloned DNA sequence encoding the 1879-2267 peptide

<220>

<221> CDS

<222> (1)..(1164)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (529)..(531)

<223> encodes S2056 residue

<400> 19

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ttccatggct cgtgtattac agaaggaaat gaacttacia agacattgat taaattgtgc      120
tacgatgcat ttacagagaa catggcagga gagaatcagc tgctggagag gagaagactt      180
taccattgtg cagcatacaa ctgcgccata tctgtcatct gctgtgtctt caatgagtta      240
aaattttacc aagggttttct gtttagtgaa aaaccagaaa agaacttgct tattttttgaa      300
aatctgatcg acctgaagcg ccgctataat tttcctgtag aagttgaggt tcctatggaa      360
agaaagaaaa agtacattga aattaggaaa gaagccagag aagcagcaaa tggggattca      420
gatggtcctt cctatatgtc ttccctgtca tatttggcag acagtaccct gagtgaggaa      480
atgagtcaat ttgatttctc aaccggaggt cagagctatt catacagctc ccaagaccct      540
agacctgcca ctggtcgttt tcggagacgg gagcagcggg accccacggt gcatgatgat      600
gtgctggagc tggagatgga cgagctcaat cggcatgagt gcatggcgcc cctgacggcc      660
ctgggtcaagc acatgcacag aagcctgggc ccgcctcaag gagaagagga ttcagtgcca      720
agagatcttc cttcttggat gaaattcctc catggcaaac tgggaaatcc aatagtacca      780
ttaaatatcc gtctcttctt agccaagctt gttattaata cagaagaggt ctttcgccct      840
tacgcgaagc actggcttag ccccttgctg cagctggctg cttctgaaaa caatggagga      900
gaaggaattc actacatggt ggttgagata gtggccacta ttctttcatg gacaggcttg      960
gccactccaa caggggtccc taaagatgaa gtgttagcaa atcgattgct taatttccta     1020
atgaaacatg tctttcatcc aaaaagagct gtgttttagac acaaccttga aattataaag     1080
acccttgtcg agtgctggaa ggattgttta tccatccctt ataggttaat atttgaaaag     1140

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ttttccggt aagatcctaa ttct

1164

<210> 20

<211> 2463

<212> DNA

<213> Artificial Sequence

<220>

<223> cDNA sequence encoding the 1879-2700 peptide

<220>

<221> CDS

<222> (1)..(2463)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (529)..(531)

<223> encodes S2056 residue

<220>

<221> misc_feature

<222> (2188)..(2190)

<223> encodes T2609 residue

<400> 20

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ttccatggct cgtgtattac agaaggaaat gaacttaca agacattgat taaattgtgc	120
tacgatgcat ttacagagaa catggcagga gagaatcagc tgctggagag gagaagactt	180
taccattgtg cagcatacaa ctgcgccata tctgtcatct gctgtgtctt caatgagtta	240
aaattttacc aagggtttct gtttagtgaa aaaccagaaa agaacttgct tatttttgaa	300
aatctgatcg acctgaagcg ccgctataat tttcctgtag aagttgaggt tcctatggaa	360
agaaagaaaa agtacattga aattaggaaa gaagccagag aagcagcaaa tggggattca	420
gatggtcctt cctatatgtc ttccctgtca tatttggcag acagtaccct gagtgaggaa	480
atgagtcaat ttgatttctc aaccggaggt cagagctatt catacagctc ccaagaccct	540
agacctgcca ctggtcgttt tcggagacgg gagcagcggg accccacggt gcatgatgat	600
gtgctggagc tggagatgga cgagctcaat cggcatgagt gcatggcgcc cctgacggcc	660
ctgggtcaagc acatgcacag aagcctgggc ccgcctcaag gagaagagga ttcagtgcc	720
agagatcttc cttcttggt gaaattcctc catggcaaac tgggaaatcc aatagtacca	780
ttaaatatcc gtctcttctt agccaagctt gttattaata cagaagaggt ctttcgccct	840
tacgcgaagc actggcttag ccccttgctg cagctggctg cttctgaaaa caatggagga	900
gaaggaattc actacatggt ggttgagata gtggccacta ttctttcatg gacaggcttg	960

gccactccaa caggggtccc taaagatgaa gtgttagcaa atcgattgct taatttccta 1020
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tataagatga tgccaaagtt aaaaccagta gaactccgag aacttctgaa ccccgttgtg 1740
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ctggcactaa attccttata ttctcctaag atagaagtgc actttttaag tttagcaaca 2040
aattttctgc tcgaaatgac cagcatgagc ccagattatc caaaccccat gtctgagcat 2100
cctctgtcag aatgcgaatt tcaggaatat accattgatt ctgattggcg tttccgaagt 2160
actgttctca ctccgatgtt tgtggagacc caggcctccc agggcactct ccagaccctg 2220
accaggaag ggtccctctc agctcgctgg ccagtggcag ggagataag ggccaccag 2280
cagcagcatg acttcacact gacacagact gcagatggaa gaagctcatt tgattggctg 2340
accgggagca gcactgacct gctggctgac cacaccagtc cctcatctga ctcttgctg 2400
tttcccaca agaggagtga aaggttacag agagcaccct tgaagtcagt ggggcctgat 2460
ttt 2463

<210> 21

<211> 1320

<212> DNA

<213> Artificial sequence

<220>

<223> cDNA sequence encoding the 2261-2700 peptide

<220>

<221> CDS

<222> (1)..(1320)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (1045)..(1047)

<223> encodes T2609 residue

<400> 21

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atggccaatg acctgcctcc ctatgacca cagtgtggca tccagagtag cgaatacttc      120
caggcttttg tgaataatat gtcctttgta agatataaag aagtgtatgc cgctgcagca      180
gaagttctag gacttatact tcgatatgtt atggagagaa aaaacatact ggaggagtct      240
ctgtgtgaac tggttgcgaa acaattgaag caacatcaga atactatgga ggacaagttt      300
attgtgtgct tgaacaaagt gaccaagagc ttccctcctc ttgcagacag gttcatgaat      360
gctgtgttct ttctgctgcc aaaatttcat ggagtgttga aaacactctg tctggagggtg      420
gtactttgtc gtgtggaggg aatgacagag ctgtacttcc agttaagag caaggacttc      480
gttcaagtca tgagacatag agatgatgaa agacaaaaag tatgtttgga cataatttat      540
aagatgatgc caaagttaaa accagtagaa ctccgagaac ttctgaaccc cgttgtggaa      600
ttcgtttccc atccttttac aacatgtagg gaacaaatgt ataatttct catgtggatt      660
catgataatt acagagatcc agaaagtgag acagataatg actcccagga aatattttaag      720
ttggcaaaag atgtgctgat tcaaggattg atcgatgaga accctggact tcaattaatt      780
attcgaaaatt tctggagcca tgaaactagg ttaccttcaa ataccttga cgggttgctg      840
gcactaaatt ccttatattc tcctaagata gaagtgcact ttttaagttt agcaacaaat      900
tttctgctcg aaatgaccag catgagccca gattatccaa accccatgtt cgagcatcct      960
ctgtcagaat gcgaatttca ggaatatacc attgattctg attggcgttt ccgaagtact     1020
gttctcactc cgatgtttgt ggagaccag gcctcccag gcactctcca gaccgtacc     1080
caggaagggg ccctctcagc tcgctggcca gtggcagggc agataagggc caccagcag     1140
cagcatgact tcacactgac acagactgca gatggaagaa gtcatttga ttggctgacc     1200
gggagcagca ctgaccgct ggtcgaccac accagtcctt catctgactc cttgctgttt     1260
gccacaaga ggagtgaag gttacagaga gcacccttga agtcagtggt gcctgatttt     1320
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<210> 22

<211> 600
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> cDNA sequence encoding the 2500-2700 peptide

<220>
 <221> CDS
 <222> (1)..(600)
 <223> HUMAN GENETIC ORIGIN

<220>
 <221> misc_feature
 <222> (325)..(327)
 <223> encodes T2609 residue

<400> 22

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ttggcaaaag atgtgctgat tcaaggattg atcgatgaga accctggact tcaattaatt      60
attcgaaaatt tctggagcca tgaaactagg ttaccttcaa ataccttgga ccggttgctg    120
gcactaaatt ccttatattc tcctaagata gaagtgcact ttttaagttt agcaacaaat    180
tttctgctcg aaatgaccag catgagccca gattatccaa accccatggt cgagcatcct    240
ctgtcagaat gcgaatttca ggaatatacc attgattctg attggcgttt ccgaagtact    300
gttctcactc cgatgtttgt ggagaccag gcctcccagg gcactctcca gacccgtagc    360
caggaagggt ccctctcagc tcgctggcca gtggcagggc agataagggc caccagcag    420
cagcatgact tcacactgac acagactgca gatggaagaa gtcatttga ttggctgacc    480
gggagcagca ctgaccgct ggtcgaccac accagtccct catctgactc cttgctgttt    540
gccacaaga ggagtgaaag gttacagaga gcacccttga agtcagtggg gcctgatttt    600

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<210> 23

<211> 1284
 <212> DNA
 <213> Artificial sequence

<220>
 <223> cDNA sequence encoding 2275-2702 peptide

<220>
 <221> CDS
 <222> (1)..(1284)
 <223> HUMAN GENETIC ORIGIN

<220>
 <221> misc_feature
 <222> (1003)..(1005)
 <223> encodes T2609 residue

<400> 23

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caattgctag gcacgtgat ggccaatgac ctgcctccct atgacccaca gtgtggcatc      60
cagagtagcg aatacttcca ggctttggtg aataatatgt cctttgtaag atataaagaa    120
gtgtatgccg ctgcagcaga agttctagga cttatacttc gatatgttat ggagagaaaa    180
aacatactgg aggagtctct gtgtgaactg gttgcgaaac aattgaagca acatcagaat    240
actatggagg acaagtttat tgtgtgcttg aacaaagtga ccaagagctt ccctcctctt    300
gcagacaggt tcatgaatgc tgtgttcttt ctgctgcaa aatttcatgg agtggtgaaa    360
acactctgtc tggagggtgt actttgtcgt gtggaggga tgacagagct gtacttccag    420
ttaaagagca aggacttcgt tcaagtcatg agacatagag atgatgaaag acaaaaagta    480
tgtttgga caaatttataa gatgatgcca aagttaaaac cagtagaact ccgagaactt    540
ctgaaccccg ttgtggaatt cgtttcccat cttctacaa catgtaggga acaaatgtat    600
aatattctca tgtggattca tgataattac agagatccag aaagtgagac agataatgac    660
tcccaggaaa tatttaagtt ggcaaaagat gtgctgattc aaggattgat cgatgagaac    720
cctggacttc aattaattat tcgaaatttc tggagccatg aaactagggt accttcaaat    780
accttggacc ggttgctggc actaaattcc ttatattctc ctaagataga agtgcacttt    840
ttaagtttag caacaaattt tctgctcgaa atgaccagca tgagcccaga ttatccaaac    900
cccatgttcg agcatcctct gtcagaatgc gaatttcagg aatataccat tgattctgat    960
tggcgtttcc gaagtactgt tctcactccg atgtttgtgg agaccaggc ctcccagggc   1020
actctccaga ccgtaccca ggaaggggtc ctctcagctc gctggccagt ggcagggcag   1080
ataagggcca ccagcagca gcatgacttc acactgacac agactgcaga tggaagaagc   1140
tcatttgatt ggctgaccgg gagcagcact gaccgctgg tcgaccacac cagtccttca   1200
tctgactcct tgctgtttgc ccacaagagg agtgaaaggt tacagagagc acccttgaag   1260
tcagtggggc ctgatttttg gaaa                                     1284

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<210> 24

<211> 819

<212> DNA

<213> Artificial Sequence

<220>

<223> cDNA sequence encoding 2429-2072 peptide

<220>

<221> CDS

<222> (1)..(819)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (538)..(540)
 <223> encodes T2609 residue

<400> 24

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gaaagacaaa aagtatgttt ggacataatt tataagatga tgccaaagtt aaaaccagta      60
gaactccgag aactttctgaa ccccggtgtg gaattcgttt cccatccttc tacaacatgt      120
agggaaacaaa tgtataatat tctcatgtgg attcatgata attacagaga tccagaaagt      180
gagacagata atgactccca ggaaatattt aagttggcaa aagatgtgct gattcaagga      240
ttgatcgatg agaaccctgg acttcaatta attattcgaa atttctggag ccatgaaact      300
aggttacctt caaataacctt ggaccgggtg ctggcactaa attccttata ttctcctaag      360
atagaagtgc actttttaag tttagcaaca aattttctgc tcgaaatgac cagcatgagc      420
ccagattatc caaaccccat gttcgagcat cctctgtcag aatgcgaatt tcaggaatat      480
accattgatt ctgattggcg tttccgaagt actgttctca ctccgatgtt tgtggagacc      540
caggcctccc agggcactct ccagaccctg acccaggaag ggtccctctc agctcgctgg      600
ccagtggcag ggcagataag ggccaccag cagcagcatg acttcacact gacacagact      660
gcagatggaa gaagctcatt tgattggctg accggggagca gcactgaccc gctggtcgac      720
cacaccagtc cctcatctga ctcttctgtg tttgccaca agaggagtga aaggttacag      780
agagcaccct tgaagtcagt ggggcctgat tttgggaaa      819
  
```

<210> 25

<211> 420

<212> DNA

<213> Artificial Sequence

<220>

<223> cDNA Sequence encoding 2561-2700 peptide

<220>

<221> CDS

<222> (1)..(420)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (145)..(147)

<223> Encodes T2609 residue

<400> 25

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tttctgctcg aaatgaccag catgagccca gattatccaa accccatgtt cgagcatcct      60
ctgtcagaat gcgaatttca ggaatatacc attgattctg attggcgttt ccgaagtact      120
gttctcactc cgatgtttgt ggagaccag gcctcccagg gcactctcca gaccctgacc      180
  
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caggaagggt ccctctcagc tcgctggcca gtggcagggc agataagggc caccagcag 240
 cagcatgact tcacactgac acagactgca gatggaagaa gctcatttga ttggctgacc 300
 gggagcagca ctgacccgct ggtcgaccac accagtcctt catctgactc cttgctgttt 360
 gcccacaaga ggagtgaaag gttacagaga gcacccttga agtcagtggg gcctgatttt 420

<210> 26

<211> 306

<212> DNA

<213> Artificial Sequence

<220>

<223> cDNA sequence encoding 2600-2702 peptide

<220>

<221> CDS

<222> (1)..(306)

<223> HUMAN GENETIC ORIGIN

<220>

<221> misc_feature

<222> (25)..(27)

<223> Encodes T2609 residue

<400> 26

gttctcactc cgatgtttgt ggagaccag gcctcccagg gcactctcca gaccgtacc 60
 caggaagggt ccctctcagc tcgctggcca gtggcagggc agataagggc caccagcag 120
 cagcatgact tcacactgac acagactgca gatggaagaa gctcatttga ttggctgacc 180
 gggagcagca ctgacccgct ggtcgaccac accagtcctt catctgactc cttgctgttt 240
 gcccacaaga ggagtgaaag gttacagaga gcacccttga agtcagtggg gcctgatttt 300
 gggaaa 306

<210> 27

<211> 34

<212> DNA

<213> Artificial sequence

<220>

<223> Primer to create T2609A mutation

<400> 27

tccgatgttt gtggaggacc aggcctccca gggc 34

<210> 28

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Reverse primer to create T2609A mutation

<400> 28

gccctgggag gcctggtcct ccacaaacat cgga

34

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/12380

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 16/40; C12N 9/12; G01N 33/53, 33/573

US CL : 435/7.1, 7.4, 194; 530/388.26

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 7.4, 194; 530/388.26

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DOUG W. CHAN et al. Ku-Dependent Phosphorylation of the DNA-Dependent Protein Kinase Catalytic Subunit is Required for the Repair of DNA Double Strand Breaks. Ataxia-Telangiectasia and ATM, 2002, page 16, see entire document.	1-4,9-18
Y	STEVEN M. YANNONE et al. Werner Syndrome Protein is Regulated and Phosphorylated by DNA-Dependent Protein Kinase. The Journal of Biological Chemistry. 12 October 2001, Vol. 276, No. 41, pages 38242-38248, see entire document.	1-4,9-18

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

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Date of the actual completion of the international search

21 September 2003 (21.09.2003)

Date of mailing of the international search report

30 SEP 2003

Name and mailing address of the ISA/US

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Commissioner for Patents

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INTERNATIONAL SEARCH REPORT

PCT/US03/12380

Continuation of B. FIELDS SEARCHED Item 3:

WEST 2.1, MEDICINE/BIOTECH (compendium databases on DIALOG) search terms: inventor names, dna pk, dna dependent protein kinase, phosphoryl?, autophosphoryl?, thr, threonine, ser, serine 2609, 2056, t2609, s2056, ser2056, serine2056, threonine2609, thr2609